Experiments and models to understand gene flow from transgenic fish in different environments

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Dedication

"Let no one tell you the medaka is uninteresting and unbeautiful. And so far as good qualities for the aquarium go, the medaka can challenge even such a redoubtable fish as the guppy! Champion of the egglayers, I call the medaka!"

- Myers 1952: p. 194

My research was made possible by the Japanese medaka (*Oryzias latipes*). Medaka are truly the fruit fly of the vertebrate world, having been extensively exploited for genetics, developmental biology, physiology, and other sciences (Yamamoto 1975). In spite of their significance in Japan (e.g., Iwamatsu 2002), very little is known, and even less has been translated into English, about the natural history of this diminutive fish. Many medaka have sacrificed their lives for my research, and I dedicate this haiku, and my dissertation, to the medaka:

Better known in labs
than in their own habitat
Orphans of science

- Kelly Pennington
January 2010, Saint Paul, Minnesota, USA

Abstract

Transgenic fishes are nearing commercialization for aquaculture around the world. Farmed transgenic fish would likely escape from typical production facilities and interbreed with wild relatives. We tested methodologies for predicting the risk of gene flow from transgenic fish.

We conducted the first study of gene flow in confined populations of transgenic animals. In two experiments several generations long, we released growth-enhanced transgenic (T) Japanese medaka (*Oryzias latipes*) into populations of wild-type (W) medaka in semi-natural environments. Transgene frequencies varied in the first experiment, but transgene frequencies all decreased in the second experiment. We measured six fitness traits in both genotypes, and found that T males were more fertile than W, but W males obtained more matings than T males.

Next, we compared fitness traits of W and T medaka under four environments: (A) high food availability, predation absent; (B) high food availability, predation present; (C) low food availability, predation absent; and (D) low food availability, predation present. Overall, T females were more fecund than W, and fecundity was highest in Environment B. Offspring of TW and WT crosses had higher survival to sexual maturity than offspring of two W parents. Fish in Environment A reached sexual maturity sooner than fish in all other environments. W males had a mating advantage in Environments B and C.

Finally, we observed gene flow in populations of T and W medaka in Environments A-D for 210 days. The final transgene frequency in Environment A was greater than in Environments C or D. We parameterized a demographic model with fitness trait values collected under the same environments, which predicted that transgene frequency in Environment A would be the highest, but also overestimated transgene frequency compared to observed results. Predicted transgene frequencies overlapped with observations in Environments B and C but not in the more extreme Environments A and D.

Our results suggest that risk assessment of gene flow from T to W fish ought to consider the impact of limiting environmental factors on fitness components. Before using models to inform ecological risk assessments, predictions should be confirmed with data collected under relevant environmental conditions.

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CHAPTER 1

Introduction

Gene flow from transgenic fish

Genetically engineered fish are the first transgenic animal to be approved for sale in the United States (Knight 2003). Although the precedent-setting fluorescent GloFish® is intended for ornamental use only, other genetically engineered fish, with a variety of transgenic traits, are being developed for aquaculture around the world (Nam et al. 2007). Growth-enhanced transgenic Atlantic salmon (*Salmo salar*) are approaching commercialization for large-scale aquaculture (National Research Council 2002; Aqua Bounty Technologies 2008; Food and Drug Administration 2009). These transgenic salmon are engineered with a growth hormone gene linked to powerful promoters, which allows them to reach market size up to a year faster than wild-type salmon (Hew and Fletcher 2001).

Aquaculture is the fastest-growing sector of animal production globally (Food and Agriculture Organization of the United Nations 2003), and fish have been ranked second only to insects in their ability to escape, disperse, and become feral (National Research Council 2004). Aquaculture of salmon in net pen facilities in coastal waters, often sympatric to wild populations of salmon, is common (e.g., Naylor et al. 2005). Such farms frequently suffer escapes of thousands to hundreds of thousands of fish (e.g., Carr et al. 1997; Gross 1998) that subsequently interbreed with local conspecifics (Crozier 1993; Clifford et al. 1998). First- and advanced-generation hybrids of wild-type fish with escaped farmed fish have been found to exhibit maladaptive behaviors and depressed fitness (National Research Council 2002, 2004). Atlantic salmon is not the only transgenic aquatic organism that is nearing commercialization: a suite of aquatic species

have been genetically engineered for a variety of traits, including genetically engineered carp (*Cyprinus carpio*) and Nile tilapia (*Oreochromis niloticus*) being considered for rearing in China and Africa, respectively – the centers of diversity for wild-type fish of those species (Kapuscinski 2005).

Genetically engineered fish raised in insecure farm facilities sympatric to wild populations of conspecifics would likely escape and mate with wild relatives, which could lead to introgression or purging of the transgene from the wild population. Risk is the product of the probability of harm × probability of exposure (Oberhauser and Rivers 2003). In this scenario, the likelihood that wild fish stocks will be exposed to transgenes from genetically engineered farmed fish (i.e., the probability of exposure) is high. The critical conservation question regarding the use of genetically engineered fish for commercial production, then, is not whether the transgene will escape, but whether it would persist or spread in the population once it has escaped. Genetically engineered fish usually possess some novel advantage over wild-type fish (Muir and Howard 2004); for example, growth enhancement and higher feed conversion efficiencies to reduce time and feed inputs to raise a fish to market size (Devlin et al. 2004a). In a natural environment, such advantages could serve to remove otherwise limiting factors, such as food availability (Muir and Howard 2004). Therefore, regulators and risk assessors would benefit from a scientifically sound method for predicting the probability and fate of transgene flow to wild populations (e.g., Hallerman 2002; Kapuscinski et al. 2007).

Models to predict gene flow

Simulation models are one way of exploring gene flow from transgenic fish without actually releasing the fish into wild populations for study. The "net fitness model" is one such model. Based on classical genetics work by Prout (1971a, 1971b) that measured various fitness components of fruit flies (*Drosophila melanogaster*) to predict the relative gene frequency of two inbred lines in an introgressed population, the net fitness approach adapted Prout's work to predict the extent and population consequences of gene flow from genetically engineered fish (Muir and Howard 2001). The net fitness model uses data collected on six fitness components representing life history characteristics that determine whether or not an organism will pass on its genes: juvenile viability, age at sexual maturity, female fecundity, male fertility, relative male mating success, and adult viability (Muir and Howard 2001). These data are collected for individuals of each genotype of interest and the model uses the joint effect of these component traits, in addition to the numbers of fish of each genotype to determine the fate of a transgene and the population size of an introgressed population after a given number of generations.

Following simulations of this model, outputs have been broadly characterized into three scenarios: (1) purge, in which the net fitness of the transgenic fish is lower relative to that of the wild-type fish, and the transgene disappears from the population in a certain number of generations with no effect on final population size; (2) spread, in which the net fitness of the transgenic fish is higher relative to that of the wild-type fish, and the transgene spreads throughout the population in a certain number of generations with no

effect on final population size; and (3) the "Trojan gene" scenario, in which antagonistic effects of the transgene lead to an initial spread of the gene into the population but ultimately results in a decrease in population size (Howard et al. 2004; Muir and Howard 1999; Muir and Howard 2001). In model simulations, the Trojan gene effect is most likely to occur when transgenic male fish enjoy a high relative mating advantage but transgenic offspring suffer from a moderate reduction in juvenile viability (Muir and Howard 1999). However, the Trojan gene effect can also be triggered by combinations of: (1) increased transgenic male mating advantage and reduced transgenic adult viability; (2) increased transgenic adult viability and reduced transgenic male fertility; and (3) a reduction in transgenic male fertility coupled with an increase in both adult viability and male mating success among transgenic fish (Muir and Howard 2002a). Because it raises the possibility of extinction in an introgressed population, the Trojan gene scenario is the outcome of greatest conservation concern.

Experiments to test the net fitness model's predictive ability have been deemed high priority for use in the risk assessment of genetically engineered organisms (Kapuscinski et al. 2007). This dissertation represents the first attempt to confirm the predictions of a net-fitness type model with an invasion of transgenic fish into a population of wild-type conspecifics in confined environments.

Use of models

Although the use of models to understand populations is not new, scientists have recently embarked on a fling with modeling that has, in the opinion of some observers,

"induced an asymmetry into ecology that has been nearly fatal" (Krebs 2003). Numerous caveats have been offered about over-reliance on models in general (e.g., Oreskes et al. 1994; Scheffer and Beets 1994) and interpretation of the net fitness model in particular (Muir and Howard 2004).

Complex ecological and demographic models are often "tuned" using parameters estimated from field data and then used to make deterministic predictions into the future. More useful to risk assessors may be simple models that have the explicit purpose of serving a heuristic, or descriptive, role rather than a predictive one (Oreskes et al. 1994; Scheffer and Beets 1994). In other words, models that help us to understand what *could* happen, and why, may be more useful in the context of risk decision-making than models that assert what *will* happen.

Fitness

At the scale of an individual organism, fitness is the sum of all its developmental and physiological processes sometimes defined as the probability that a particular genotype will survive and reproduce (Klug & Cummings 1993). As a population measure, mean fitness of a population is often discussed with the assumption that the population is not constrained by the carrying capacity of its environment (Falconer 1989). However, fitness is by no means a simple concept; as Falconer (1989: p. 336) understates: "there are difficulties in defining fitness precisely." Rosenberg (1983) argues from the perspective of the philosophy of science that fitness is an elusive concept: "the hypothesis that an organism, in a given environment, has a given level of fitness is like

the hypothesis that a given die is a fair one" (Rosenberg 1983: p. 461), because an investigator could never prove this hypothesis incorrect, no matter how many replicates are used. More practically, Nakajima (1998) points out that most population genetics experiments study "mere Mendelian population[s] floating free from its external world" (Nakajima 1998: p. 314) and argues for the contextualization of fitness in a particular ecological situation with variables such as resource limitation and predator pressure. In recognition of Nakajima's (1998) point, the experiments presented in Chapters 3 and 4 incorporate two limiting ecological variables in their design.

I hope to avoid further semantic discussion of fitness by operationally defining the term in the context of my research. In this dissertation I use "fitness component measurement," "fitness trait," or similar language, to describe a life history trait with some measurable value, always in the context of a particular genotype and environmental condition.

Genotype × environment interaction

The net fitness model (Muir and Howard 2001) has certain limitations. For example, it is based on the assumption that the six fitness components will account for all the underlying physical, biochemical, and other traits that affect the fitness of an organism, and the fate of the transgene can be predicted based on these parameters alone (Muir and Howard 2004). Of greatest bearing on my research is the fact that the net fitness model has not taken into account genotype × environment (G × E) interactions that could change the values of fitness components in a wild environment (Muir and

Howard 2004). Genotype × environment interactions can result in changing ranks between genotypes or simply changing magnitude in the difference between two genotypes when measured across two or more different environments (Dunham 2004). Genotype × environment interactions have been observed in growth-hormone transgenic fish, especially in environments with different levels of food availability and predation (Bessey et al. 2004; Devlin et al. 2004b; Sundström et al. 2009). Because of the importance of genotype × environment interactions in aquaculture, Muir and Howard qualified their model's assumption by recommending that fitness measurements be collected under the environmental conditions relevant to that genetically engineered organism (Muir and Howard 2004). Understanding how fitness components might be affected by different environmental conditions would be useful for interpreting future results obtained from the net fitness model.

Model organism: Japanese medaka

The medaka is a freshwater cyprinodont native to Japan, Korea, Taiwan, and China (Yamamoto 1975). Medaka inhabit slow-moving streams and shallow pools, including rice paddies, in Japan (Iwamatsu 2002; Sawara 2003). Medaka have been reared in captivity for centuries: by the Edo Period in Japan medaka were a popular ornamental fish and methods had been developed to rear and breed them in clay pots (Iwamatsu 2002).

Contemporary medaka are more common in labs than in home aquaria; they are an ideal model organism as they are small (< 40 mm; Jiménez 2000), easy to rear and

breed under laboratory conditions, extremely hardy, and tolerant of a range of temperature, salinity, and oxygen conditions (Briggs and Egami 1959; Myers 1952). Medaka thrive on commercial flake food diets in captivity, and augmentation with a live food such as juvenile brine shrimp (*Artemia* spp.) seems to increase their overall vigor.

Medaka are well suited to fitness experiments because they are sexually dimorphic and reliable breeders. Female and male medaka are distinguished by secondary sexual characteristics such as body and fin shape (Yamamoto 1975). Medaka are iteroparous and can breed for months on end under the correct light regime (Briggs and Egami 1959); at least 13 hours of light per day are required for secretion of growth hormone from the pituitary gland and subsequent development of gonads (Iwamatsu 2002). Eggs are released upon copulation (which usually occurs in the "morning," or shortly after the lights come on), fertilized externally, and remain attached to the female's oviduct by filamentous threads until she brushes the egg mass onto vegetation or another surface (Yamamoto 1975).

My research used two different genotypes of Japanese medaka, *Oryzias latipes*. I identify these two genotypes in the dissertation as "wild-type" (abbreviated W) and "transgenic" (T). The wild-type medaka have not been genetically engineered, and are indeed thought to be a nearly wild strain of fish with a relatively high background genetic diversity. We obtained the wild-type medaka from a breeder in Southeast Asia through an American dealer (Pacific Aquatics, Chatsworth, CA, USA). To create transgenic medaka, wild-type medaka from Japan were genetically engineered using microinjection (Jiménez 2000) with a rainbow trout (*Oncorhynchus mykiss*) metallothionein promoter sequence

and a sockeye salmon (*Oncorhynchus nerka*) growth hormone gene (pOnMtGH1) (for construct, see Devlin et al. 2004a). Transgenic medaka of the sGH-400 (T400) and sGH-67 (T67) line were generously provided by Dr. William Muir (Purdue University) who obtained the transgene construct from Dr. Robert Devlin (University of British Columbia). In Chapter 2, both lines of transgenic medaka are used, and are identified as T400 and T67; in Chapters 3 and 4, the T67 line is the only transgenic line in use and is identified simply as T. From 2001-2009, we maintained W and T medaka in the Aquaculture and Fisheries Laboratory on the University of Minnesota St. Paul campus, and I cared for them in accordance with husbandry and containment procedures approved the University of Minnesota's Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee (IBC).

Combining fitness and gene flow experiments with models to understand gene flow from transgenic fish

I wrote Chapters 2, 3, and 4 of this dissertation as manuscripts to submit to peer-reviewed scientific journals for publication. Therefore, these chapters are written in first-person plural "we" instead of singular "I."

In Chapter 2, "Multi-generational gene flow trials consistent with fitness differences in transgenic and wild-type Japanese medaka fish (*Oryzias latipes*)," I present results from the first multigenerational test of gene flow from transgenic to wild-type fish. I discuss the results of two experiments in which growth-enhanced transgenic medaka "invaded" populations of wild-type medaka in semi-natural environments. We

also measured selected fitness traits in transgenic and wild-type medaka. Our fitness measurement results, showing a lack of transgenic advantage, are consistent with our invasion experiment results, and support the use of fitness trait data to predict gene flow and its population effects. Future application of this approach to real risk assessments would require collecting fitness trait data under environmental conditions that capture the variability in key ecological factors in the ecosystems at issue.

In Chapter 3, "Predation and food limitation influence fitness traits of growth-enhanced transgenic and wild-type fish," I compare selected fitness traits of wild-type (W) and growth-enhanced transgenic (T) medaka measured under four different environmental conditions: (A) high food availability, predation absent; (B) high food availability, predation present; (C) low food availability, predation absent; and (D) low food availability, predation present. I conclude that, based on our results, future risk assessments of gene flow from transgenic to wild relatives must incorporate ecosystem-specific variability in limiting ecological factors and their potential impact on fitness components of different genotypes.

Chapter 4, "Experimental measurement and stochastic modeling of multi-generational gene flow from transgenic to wild fish under varying environments," combines the lessons learned in Chapters 2 and 3 to report on multigenerational gene flow experiments we conducted under the four different environments used in Chapter 3. I also discuss the predictions of deterministic and stochastic versions of a population model to describe gene flow under those environments, parameterized with the fitness measurements taken in Chapter 3. I found that while models based on fitness component

measurements may hold promise for predicting gene flow, caution must be exercised when applying models to ensure that they are confirmed under relevant environmental conditions.

CHAPTER 2

Multi-generational gene flow trials consistent with fitness differences in transgenic and wild-type Japanese medaka fish (*Oryzias latipes*)

with Anne R. Kapuscinski, Michael S. Morton, Anne M. Cooper, Loren M. Miller, and William M. Muir

Summary

Transgenic fish in development for aquaculture could escape from fish farms and interbreed with wild relatives in the nearby environment. Predicting whether escapes would result in transgene introgression is one of the major challenges in assessing environmental risks of transgenic fish. Measurement of fitness-related traits can be used to predict the relative selective value of transgenic genotypes, and the potential for gene flow. Although studies have simulated gene flow from transgenic fish using mathematical models, ours is the first to study gene flow in confined populations of transgenic animals. We conducted two invasion experiments in which we released growth-enhanced transgenic fish, Japanese medaka (*Oryzias latipes*), into populations of wild-type medaka in semi-natural environments. After several generations, transgene frequencies varied across replicates in the first experiment, but transgene frequencies all decreased in the second experiment. We also measured selected fitness traits in transgenic and wild-type medaka. Overall, transgenic males were more fertile than wild-types. Offspring of wild-type females lived significantly longer than those with transgenic mothers. Wild-type males obtained more matings with females than transgenic males. Our results contrast with previous findings that growth-enhanced transgenic medaka have a mating advantage; because we used a different strain of wild-type fish than these studies, genetic background effects could account for our results. The decreasing transgene frequency in the second invasion experiment indicates that transgenic fish may have a selective disadvantage in the experimental environment. Our fitness measurement results, showing a lack of transgenic advantage, are consistent with our invasion experiment results and

support the use of fitness trait data to predict gene flow and its population effects. Future application of this approach to real risk assessments would require collecting fitness trait data under environmental conditions that capture the variability in key ecological factors in the ecosystems at issue.

Introduction

Farmed fish are known to escape from farms in large numbers and with a high frequency, and to hybridize with wild fish (National Research Council 2004). Gene flow from escaped farmed salmon to wild salmon populations has been shown to depress fitness, which can reduce local adaptation and productivity and, under continual gene flow, prevent re-adaptation of the wild population (Hindar et al. 1991; McGinnity et al. 2003; Naylor et al. 2005). Such gene flow can lead to rapid genetic homogenization between the farmed and wild populations, reducing the long-term ability of wild populations to adapt to environmental change (Hindar et al. 1991; Fleming et al. 2000). In addition, the expression of novel phenotypes by transgenic fish could result in different fitness-related traits compared to wild relatives, with population-wide consequences. In the case of growth-enhanced transgenic fish, the effect of mechanisms like resource competition might be amplified by the higher feeding motivation of faster-growing fish (Johnsson et al. 1996; Jönsson et al. 1996; Sundström et al. 2003). Farmed transgenic fish are expected to escape from aquaculture systems and interbreed with wild relatives, unless they are raised under adequate physical and biological confinement (National Research Council 2004).

Predicting gene flow from escaped transgenic fish to a wild population is a major need in environmental risk assessment of transgenic fish (Devlin et al. 2006; Kapuscinski et al. 2007; National Research Council 2008). The timeliness of this issue is illustrated by the fact that growth-enhanced transgenic Atlantic salmon (*Salmo salar*) are approaching commercialization for large-scale aquaculture (National Research Council 2002; Aqua Bounty Technologies 2008; Food and Drug Administration 2009). Life-history traits affecting fitness are one way of predicting the potential invasiveness of a species (e.g., Sakai et al. 2001), though correlations between more invasive fish species and life history characteristics favoring a fitness advantage are only recently being tested (i.e., Vila-Gispert et al. 2005). Laboratory studies have found that growth-enhanced transgenic fish and their unmodified relatives can have different values for some primary components of total fitness, such as increased male mating advantage (Howard et al. 2004) and a moderate reduction in juvenile viability in transgenic individuals (Muir and Howard 2001). A number of studies have considered the possible results of an invasion of transgenic fish, based on relative values of selected fitness-related traits (either hypothetical or experimentally measured; e.g., Muir and Howard 1999; Hedrick 2001; Muir and Howard 2001), such as viability, fecundity, and male mating advantage. However, these studies have so far only simulated a transgene invasion with deterministic models; no studies we are aware of have attempted to study gene flow from transgenic animals in confined populations.

We used Japanese medaka fish (*Oryzias latipes*) in our study. Medaka are well-suited to population biology research (Yamamoto 1975) and can live and reproduce well

in laboratory conditions. Medaka are small (usually less than 4 cm in length), and have a generation time of about two months. Given the right conditions, medaka can mate daily for an extended period of time. Reproductive activity usually takes place within an hour of daybreak, or as simulated by the beginning of the photoperiod in captivity (Wittbrodt et al. 2002; Shima and Mitani 2004).

In addition to being a useful model for population genetics research, medaka have proven to be relatively easy to genetically engineer; in fact, one of the first reports of stable trangenesis in fish used medaka (Ozato et al. 1986). We used two lines (MtsGH-67 and MtsGH-400; hereafter T67 and T400, respectively) of growth-enhanced transgenic medaka (produced by William Muir, Purdue University) derived from a single genetic background and engineered with an all-salmonid gene construct (Devlin et al. 1994) consisting of a metallothionein-B promoter (Chan and Devlin 1993) and sockeye salmon full-length, type-1 growth hormone gene (Devlin 1993). We used a line of wild-type medaka (hereafter W) descended from wild fish captured in Japan (obtained from Pacific Aquatics, Chatsworth, CA, USA).

Our objective was to test whether the relative fitness of transgenic fish could provide useful information about the outcome of an invasion of transgenic fish into a wild-type population. In a confined laboratory environment, we carried out two types of experiments using medaka: (1) invasion experiments, in which large populations of W medaka were "invaded" by transgenic medaka; and (2) fitness trait measurements, in which we compared the fecundity, fertility, mating advantage, juvenile viability, age at sexual maturity, and longevity of W, T67 and T400 medaka. We controlled a number of

key environmental conditions across all experiments, including water temperature, feeding regime, water source, and photoperiod.

We carried out the invasion experiments using nine mesocosms, large fiberglass tanks with habitat structure. All mesocosms were stocked with large numbers of wild-type medaka. Three mesocosms were invaded by T67 medaka, three by T400 medaka, and three were maintained as control (W only) populations (Tab. 1). Invasion experiments were repeated twice: invasion experiment I ran for three generations; invasion experiment II lasted nearly ten generations.

We measured fitness traits on fish housed in glass aquaria. Fecundity and fertility were measured on wild-type and transgenic fish paired in pure and reciprocal crosses (Tab. 2). We tested T67 and T400 medaka with W fish in two trials each, resulting in a total of four trials. We used offspring from the fecundity and fertility experiments to measure age at sexual maturity, juvenile viability, and longevity. We tested the relative mating success of W males and each transgenic line separately, in five test periods for each T400 and T67 experiment.

Results

Invasion experiments

At the beginning of invasion experiment I, T67 adults were the heaviest on average (mean ± SD: 0.512 ± 0.098 g, n = 60), followed by wild-type adults (0.359 ± 0.057 g, n = 16) and T400 adults (0.322 ± 0.130 g, n = 60). We confirmed expression of the transgene in transgenic medaka of both lines (Fig. 1).

In invasion experiment I, final transgene frequency varied across replicates, ranging from disappearance of the transgene in three of the mesocosms and a decreasing transgene frequency in one mesocosm, to an increase in two of the T400 mesocosms (Fig. 2a; sign test, $p = 0.344$). In experiment II, we observed a mean transgene frequency of 0.114 (SD = 0.057) in the T67 mesocosms five months into the experiment, but a consistent decline in transgene frequency in all populations thereafter. By the end of the experiment, we found no transgenic fish in five mesocosms and only one T67 transgenic fish remained in the sixth (Fig. 2b); a significant decrease in transgene frequency (sign test, $p = 0.016$).

Reproduction and recruitment occurred in all mesocosms in both experiments. In experiment I, in the mesocosm in which the transgene frequency increased, two T400 fish at the end of the experiment were 12 and 16 mm standard length, and therefore too small to have been introduced at the initiation of the experiment. These two fish were offspring and they, along with our regular visual observation of eggs and fry in the mesocosms after the second week of both experiments, provided evidence of reproduction by both wild-type and transgenic adults.

In both experiments, population sizes decreased in each mesocosm (Fig. 3), but multiple comparisons tests revealed no significant differences in mean final population size between control and experimental treatment populations (Tukey's HSD, all $p > 0.566$). Pathological tests on a sample of the deceased experimental fish indicate that the observed declines were caused by a *Mycobacterium haemophilum* infection (unpublished

data, S. Robbe-Austerman, USDA-APHIS, National Veterinary Services Laboratories, Ames, IA).

Fitness measurements

Fecundity

Transgenic females weighed significantly more than wild-type females at the start of all fecundity trials (Tukey's HSD, T67-W and T400-W both adjusted $p < 0.000$). The interaction of male and female genotype (ANOVA, $F_{(6, 61)}$, $p = 0.376$) did not have a significant effect on total ten-day egg production.

Fertility

The overall fertility rate of all males was high, regardless of trial or genotype, with few infertile or low-fertility males. T400 males were significantly more fertile than wild-type males (Tab. 3; 95% Bootstrap CI with Bonferonni correction; W-T400: -8.342, -0.564).

Mating Advantage

In the T400 mating advantage experiments, W males obtained 53% of matings alone, and T400 males mated with the female alone 27% of the time. When we considered W matings as "successes" and T400 matings as "failures" in a binomial model, W males had a significant advantage (intercept = 0.560, $z_{(df=19)} = 2.187$, $p = 0.029$). The remaining 20% of the matings were trio matings, in which both the W and

T400 male mated with the female in the same mating event, but we did not test the paternity of the fertilized eggs. In a model to simulate a case where all trio matings were assigned to the W male, the W advantage increased (intercept = 0.677, $z_{(df = 22)} = 3.121$, p = 0.002). At the other extreme, when we combined trio matings with T400 matings, W males retained a small, but not significant, advantage (intercept = 0.105, $z_{(df = 22)} = 0.513$, p = 0.608). T400 males weighed, on average, 0.035 g more than W males in the T400 experiments; however that difference was not significant (t-test, $t_{(df = 38)} = 1.014$, p = 0.317).

In the T67 mating advantage experiments, 45% of the matings were trio matings. W males obtained 37% of matings alone, while T67 males mated with the female alone 18% of the time. A binomial model in which W matings were successes and T67 matings were failures had a significant intercept term (intercept = 0.731, $z_{(df = 15)} = 2.165$, p = 0.030). When all trio matings were assigned to the model with W successes, the W advantage increased dramatically (intercept = 1.460, $z_{(df = 23)} = 4.744$, p = 0.000). A model in which T67 and trio matings, together, were considered successes, and W matings were failures, was not significant (intercept = 0.442, $z_{(df = 23)} = 1.791$, p = 0.073). On average, T67 males were 0.039 grams heavier than the W males they were paired with, a significant size advantage (t-test, $t_{(df = 43)} = 2.873$, p = 0.006).

Age at Sexual Maturity

T67 females began to produce eggs at the earliest age (mean ± SD, 48.760 ± 6.809 days), followed by W (51.684 ± 6.225) and T400 (54.846 ± 3.209) fish, though

these differences were not significant (Tukey's HSD, all adj. $p > 0.880$). Neither the genotype of the individual (ANOVA, $F_{(2, 67)} = 0.931$, $p = 0.399$) nor the interaction between maternal and paternal genotype (ANOVA, $F_{(6, 67)} = 1.961$, $p = 0.084$) were significant predictors of age at sexual maturity.

Juvenile Viability

In trial 1, we observed 70% survival to sexual maturity among fry from two T400 parents, compared to 94% among offspring of two W parents and 98% among offspring of one W and one T400 parent. We found differences between observed and expected values across the four parental crosses in trial 1 ($\chi^2_{(df = 3)} = 30.220$, $p = 0.000$). We excluded data for trial 2 from analysis because we made errors stocking fry in that trial.

Most fish in trial 3 died at about the same time (mean ± SD, 22 ± 6.967 days), regardless of parentage. Observed and expected proportions surviving to sexual maturity did not differ by parental cross in trial 3 ($\chi^2_{(df = 3)} = 7.110$, $p = 0.069$). Neither offspring genotype (ANOVA, $F_{(1, 35)} = 0.136$, $p = 0.714$) nor the interaction between parental genotypes (ANOVA, $F_{(3, 35)} = 0.460$, $p = 0.712$) were significant. We did not detect any deceased fish before sexual maturity in trial 4.

Longevity

The average medaka in our experiments lived to be over a year old (mean ± SD, 382 ± 178 days). In the model to predict longevity of adult fish, the interaction between parental genotypes (ANOVA, $F_{(6, 356)} = 10.266$, $p = 0.000$) was significant, but genotype

of the deceased fish (ANOVA, $F_{(2, 356)} = 1.246$, $p = 0.290$) was not. Offspring of W x W parents and W x T400 parents lived significantly longer than offspring of the following crosses: T400 x T400, T400 x W, and T67 x W (all adjusted $p < 0.024$). Offspring of T67 x T67 parents also survived significantly longer than T400 x T400 offspring (adj. $p = 0.029$). W individuals lived significantly longer than T400 fish (Tukey's HSD, W-T400, $p = 0.032$), but not significantly longer than T67 fish (Tukey's HSD, W-T67, $p = 0.913$).

Discussion

The results of our invasion experiments and fitness trait measurements, measured under similar environmental conditions, were consistent. In our invasion experiments, the transgene usually decreased in frequency, and most experiments resulted in a total loss of all transgenic fish. No single genotype emerged as a clear "winner" on the basis of individual fitness trait measurements (Tab. 4). The T400 males had a significant fertility advantage over W males. However, wild-type males were more likely than T67 males to secure matings with W females. W fish lived significantly longer than T400 fish. There were no significant differences between transgenic and wild-type fish in fecundity, age at sexual maturity, or juvenile viability.

Invasion experiments

Our transgene frequency results were not wholly unidirectional; therefore, they likely reflect the combined effects of random processes and varying selection pressures, as would occur if transgenic fish escaped into a wild population. Possible mechanisms for

the observed changes in transgene frequency include: genetic drift, or random changes in frequency of the transgene, ultimately leading to its fixation or loss; demographic stochasticity; or differing selection pressures due to uncontrolled environmental variation among the mesocosms.

Genetic drift would be equally likely to result in an increased or decreased transgene frequency. Given the significant results we observed in invasion experiment II, it appears that the transgene is not a selectively neutral gene. In fact, our results imply that the transgene confers a fitness disadvantage in the environment of the mesocosms. Because invasion experiment II lasted about ten generations, we were more likely to observe fixation or loss of the transgene in that experiment; however, the tendency towards loss that we observed in all of the mesocosms does not indicate that this was a result of random drift.

To further test our gene flow results in the context of population genetics, we used the following equation to predict the number of generations to extinction of a selectively neutral mutant allele:

$$-4N/(1 - p)*(p*\ln(p)),$$

where N is the effective population size and p is the initial frequency of the mutant allele (Hartl and Clark 1997: p. 282; after Kimura and Ohta 1969). We conservatively estimated the effective population size using census data from our mesocosms: half the initial population size ($N_I = 377$ and $N_{II} = 517$) and half of the smallest final population size observed in any experimental mesocosm ($N_I = 62$ and $N_{II} = 48$). We used the starting transgene frequencies from both experiments ($p_I = 0.054$ and $p_{II} = 0.077$). Using half of

the initial population as our estimates of effective population size, the formula predicted loss of the transgene in 222 and 124 generations in invasion experiments I and II, respectively. Even the most conservative estimates, using half of the lowest final population size as the effective population size, predicted that a selectively neutral transgene would avoid loss until after an average of 21 generations for both invasion experiments. Because in invasion experiment II, the transgene disappeared from five out of six replicates in less than half that time, this simulation provides further evidence that the transgene was not selectively neutral.

We did observe the effect of an unpredictable agent of demographic change due to the *Mycobacterium haemophilum* infection. Because all mesocosms in both experiments, including wild-type-only control populations, experienced population declines at a similar rate, and of comparable magnitude, it is unlikely that the disease itself directly affected our transgene frequency results.

We monitored and kept constant most environmental factors, including temperature, photoperiod, food availability, and water chemistry, across mesocosms and in both invasion experiments. Differences in water clarity between mesocosms in experiment I might have influenced visually-affected behaviors such as mating, aggregation, and food foraging (e.g., Guthrie and Muntz 1993) leading to different selection pressures and therefore more variation in results in experiment I than in experiment II.

Fitness measurements

Fecundity

Fecundity in fish is generally positively associated with size (e.g., Jobling 1995) and previous studies using medaka have found a positive weight-fecundity relationship (Muir and Howard 2001). In spite of the fact that transgenic females weighed more than wild-type females in our experiments, we did not detect differences in fecundity between genotypes.

Fertility

T400 males had significantly greater fertility than W males in our tests. Muir and Howard (2001) did not find a difference in the fertilization success of transgenic and wild-type males.

Overall, the male fertility rates we observed in these experiments were high, except for one W male with a low fertility rate in one trial, and two transgenic males who fertilized no eggs in one trial each. We removed these data from our analyses because two of these males were tested with the same female, the third male was tested with a female that died in the middle of the trial, and total egg production during all three males' trials was very low. Our observations of high fertility rates are consistent with previous studies of male fertilization success in medaka (Muir and Howard 2001); further suggesting that the three lowest-fertility males were genuinely unusual observations.

Mating Advantage

In these experiments, both lines of transgenic males were heavier, on average, than the W males; however, W males obtained a larger proportion of matings with females. This contrasts with (1) the general assumption that larger males will have a competitive advantage in seeking matings (i.e., Andersson 1994), and (2) previous medaka research that found that larger males (Howard et al. 1998) and larger, transgenic males (Howard et al. 2004) have a mating advantage over smaller and wild-type males. However, in both of Howard et al.'s (1998 and 2004) medaka mating advantage studies, the larger males of each genotype were competing against males of the same genetic background (the Purdue orange-red strain). In our study, the transgenic males were derived from the Purdue orange-red strain, but our wild-type fish descended from medaka from Japan. The differences in genetic background between the wild-type comparators could have led to the differences in mating advantage results (e.g., Kapuscinski et al. 2007), as different strains of unmodified fish have been found to express different values for some important components of fitness (McGinnity et al. 2003).

Given that we observed a high incidence of trio matings, even a small difference in fertilization success between the first and second, or "joiner," male, could result in a substantial difference in paternity among offspring of such matings. Iwamatsu et al. (1991) suggest that most micropyles on the medaka egg are occupied by sperm after six seconds of exposure to high sperm concentrations, so it is possible that a late-joining male would fertilize a lower proportion of eggs. Howard et al. (2004) found that "joiner" male medaka fertilized about 23% of eggs in trio matings. We did not test the genotype

of eggs or offspring to determine paternity. However, we did model "worst-case" scenarios in which the males of one genotype fertilized all the eggs during trio matings. Our results suggest that even if transgenic males were fertilizing all the eggs in trio matings, they still would not have a significant overall mating advantage over wild-type males.

Age at Sexual Maturity

The fish in our experiments produced eggs at an earlier age than previously reported for wild-type medaka (i.e., 64.3 ± 1.27 days reported by Jiménez 2000). This could be due to our choice to test only the first five females in each aquarium to reach sexual maturity, as we were most interested in the earliest-maturing fish in a population.

Juvenile Viability

Our juvenile viability data are incomplete because dead fry were very difficult to detect in our experiments. In trial 1, offspring of two T400 parents were significantly less likely to survive to sexual maturity than offspring of one or no transgenic parent(s). This is consistent with Muir and Howard's (2001) study of juvenile viability to three days of age, which found offspring of two transgenic parents to have relatively lower viability.

During trial 3, most of the fish that died before reaching sexual maturity appear to have died in groups. This age range (~15-30 days old) may be a period of vulnerability and mortality due to increased competition from tank mates or another factor.

Longevity

Overall longevity in our experiments was high, with a mean lifespan of over a year. The natural lifespan of medaka is thought to be approximately one year (unpublished data, cited in Shima and Mitani 2004). Fish with W mothers had a significantly longer lifespan than transgenic medaka, and W fish survived significantly longer than T400 fish. Muir and Howard's (2001) model to predict gene flow from transgenic fish using fitness trait estimates was least sensitive to longevity, therefore differences in this fitness trait may be less likely to impact population processes like gene flow.

Conclusion

The consistency of our results between the fitness trait data and invasion experiments imply that ecological risk assessments may be able to utilize fitness trait data to predict the fate of transgenes, provided that the fitness trait data are collected under environmental conditions approximating those of the ecosystems at issue. Indeed, the aquaria we used to measure fitness traits had similar environmental conditions as those in the invasion mesocosms (i.e., our ecosystem at issue). In invasion experiment I, the transgene frequency increased in two T400-invaded populations, consistent with our findings of a T400 fertility advantage over wild-type fish. The results of invasion experiment II, at the end of which no T400 and only one T67 fish remained, are consistent with our results for longevity and mating advantage, in which wild-type fish had an advantage over T400 and T67 medaka, respectively. In most of the invasion

experiment populations, transgenic fish did not successfully invade the wild-type population.

Future research

We recommend that future transgenic gene flow experiments in animals reflect the type and degree of variation in environmental factors affecting the animal's ability to survive and reproduce in nature due to the potential fitness effects of genotype-by-environment interactions. Risk assessment scientists need to better understand how these interactions might be incorporated into risk assessments of transgenic fish. The impact of environmental variation on transgenic and wild-type fish has been considered one of the greatest challenges to our ability to predict the ecological risk of transgenic fish (Devlin et al. 2006; National Research Council 2008).

Studies of transgene flow should span many generations and include a number of replicates in order to distinguish between potentially large effects of stochastic processes and natural selection (Hill 1971). This may be impractical for species with long generation times or complex life histories (e.g., migratory salmon), but is no less important. Simulation modeling could help assess the effects of stochastic processes and selection on transgene fate, as long as parameters reflect empirical knowledge of major selection forces in the species' natural environment.

Materials and Methods

Confirmation of transgene inheritance and expression

Japanese medaka in wild-type and transgenic founder populations were housed separately according to genotype. When transgenic medaka reproduced, we used polymerase chain reaction (PCR) to determine which offspring had inherited the transgene. To obtain tissue for genetic analysis, we sampled a small (<1 mm) strip of tissue from the distal end of the caudal fin, using sterile procedure. We placed this tissue in labeled tubes containing 200 µl of a 10% Chelex solution (Sigma) and processed it according to Miller and Kapuscinski's (1996) DNA extraction protocol. We used sGH primers (SGHFOR: 5'-TCG CGC AGT ATA ATG AAA T-3' and SGHREV: 5'-TAA GAG CGC TGG GTC GTT A-3'; Integrated DNA Technologies, Coralville, IA) to identify transgene-positive fish, and we used primers for the beta-actin gene (MBAFOR: 5'-GTA GCG TAT GGG TTG GGT-3' and MBAREV: 5'-AGC AGA ATG CCA CCT CAG A-3'; Integrated DNA Technologies, Coralville, IA) as a control. Each reaction mixture contained 5 µl Chelex template DNA solution, 10 µl PCR Master Mix (Promega), 8 pmol of sGH primer and 2 pmol of beta-actin primer. We carried out PCR with 20 µl reaction mixtures, and ran PCR in a Hybaid Omn-E thermal cycler at the following temperature profile for 35 cycles: denaturing at 94 °C for 30 seconds, annealing at 50 °C for 30 seconds, and elongating at 72 °C for 30 seconds, followed by a final elongation period of 10 minutes. After electrophoresis on a 1% agarose gel stained with ethidium bromide, we visualized PCR products under ultraviolet light. Individuals were compared to lanes containing PCR product from previously confirmed wild-type

and transgenic control individuals. Transgene-negative fish were identified by a single band (beta-actin control), and transgene-positive fish were identified with two bands (beta-actin and sGH transgene).

To confirm expression of the transgene in T400 and T67 medaka, we submitted seven transgenic and one wild-type fish for reverse-transcriptase PCR (RT-PCR) at the BioMedical Genomics Center (University of Minnesota, Saint Paul, MN, USA). Total RNA was extracted from fish, homogenized in Trizol reagent (Invitrogen, CA, USA) and its quality was checked on the Agilent Bioanalyzer 2100 (Agilent Technologies, CA, USA). First strand cDNA synthesis was conducted from 2 μg of total RNA from each sample, using oligo $dT_{(12\text{-}18)}$ primers and SuperScript II reverse transcriptase (Invitrogen). Gene expression analysis was performed using SYBR Green chemistry on an Applied Biosystems 7900 Real-Time PCR system with reagents purchased from Applied Biosystems. All PCR reactions were performed in triplicate and the experiments were replicated a minimum of three times. The amplification product was checked on a 2% agarose gel.

Invasion experiments

Model population

In order to obtain a size distribution for transgenic and wild-type medaka, we weighed all introduced transgenic fish (n = 120) and a randomly selected sample of wild-type fish (n = 16) before the initiation of invasion experiment I.

Experimental design

To investigate the pattern of gene flow from transgenic to wild relatives, we released transgenic fish into populations of wild-type relatives in mesocosms with structural complexity in their environments. The mesocosms were nine 1500-liter, semi-square fiberglass tanks. To promote natural reproduction, hanging spawning grasses were provided on which females could deposit eggs. Photoperiod (16 hours light: 8 hours dark) and temperature (26 °C) were controlled and optimized for reproductive activity (Shima and Mitani 2004). Refugia were provided so that juvenile fish could voluntarily escape from cannibalistic adult medaka.

Transgenic invasion experiments were repeated twice. We conducted experiment I for the equivalent of three generations (six months). After the completion of experiment I, we conducted experiment II for nearly ten generations (nineteen months). Large populations of wild-type medaka were established in each of nine mesocosms. Three control mesocosms contained only these wild-type fish. To simulate a small escape of farmed fish (a frequent event; National Research Council 2004), we released T67 transgenic medaka into three of the nine mesocosms and T400 transgenic medaka into three others (Tab. 1). Adult fish were fed to satiation once daily with flake food (O.S.I. Marine Lab) and 24-hour-old brine shrimp (*Artemia* spp.) hatched from cysts (Aquatic Eco-Systems) in our lab. Fry in refugia were fed Artificial Plankton Rotifer (APR; O.S.I. Marine Lab).

To determine final population size and transgene frequency, we counted and genotyped all fish in each population when both invasion experiments were terminated.

In addition, we sampled fish (n = 30) from each experimental population to estimate transgene frequency at four points during invasion experiment II.

In experiment I, water clarity in the mesocosms gradually decreased as suspended algal density increased, making it difficult to identify and remove mortalities and monitor fish health. In the hopes of controlling algae in experiment II, ultraviolet sterilizers were installed on all mesocosms and succeeded in maintaining water clarity. However, fish eventually appeared stressed by the lack of cover, so we introduced two different species of floating macro-algae in equal amounts by weight across all mesocosms: duckweed (*Lemna minor*) and water sprite (*Cereus* spp.). Eight months after the initiation of experiment II, 130 g (wet weight) of duckweed was introduced to each mesocosm. In the following month, 30 g of water sprite was added to each mesocosm.

Data analysis

We used binomial sign tests to compare the final transgene frequencies in both invasion experiments. An increased final transgene frequency was considered a "success" while a transgene frequency less than the initial transgene frequency, including a result of zero transgenic fish, was considered a "failure." We performed these tests with the null hypothesis that, if the transgene is selectively neutral, the transgene frequency is equally likely to increase or decrease. We also compared ending population sizes in each mesocosm across treatments using an Analysis of Variance (ANOVA) model and Tukey's Honestly Significant Difference (HSD) multiple comparisons tests. To analyze our data, we used the open-source statistical software R (R Development Core Team,

2008), and where p-values were obtained, we set alpha = 0.05 as the threshold for significance.

Fitness trait measurements

Concurrent with the invasion experiments, we measured selected life-history components of wild-type (W) medaka and the two transgenic lines (T400 and T67): fecundity, fertility, mating advantage, age at sexual maturity, juvenile viability, and longevity.

We measured the following three fitness traits on adult fish: fecundity, male fertility, and mating advantage. We selected fish for these experiments from the same source population as the fish introduced into the invasion experiments, but we did not use the same individual fish. Fecundity and fertility data were collected in four consecutive trials: in trials 1 and 2 we measured traits of T400 fish and wild-type fish together, and four months later, in trials 3 and 4 we measured T67 fish with a different set of wild-type fish. Mating advantage experiments occurred separately, with different adult fish. We measured fecundity, fertility, and mating advantage in 5-gallon static glass aquaria with photoperiod controlled at 16:8 (light:dark) and temperature of approximately 26 °C. Adult fish were fed to satiation with flake food and live brine shrimp.

We randomly selected two consecutive days of each trial of the fecundity and fertility experiments, and retained all fertile eggs from those two days. We incubated those eggs, separated by the cross (maternal x paternal genotype) that produced them and by the trial (1-4) during which they were produced. When the eggs hatched after 8-10

days, we randomly selected 50 fry from each cross and placed them in 30-gallon glass aquaria. We used these fish to measure the remaining three fitness trait measurements: age at sexual maturity, juvenile viability, and longevity. We fed fry only APR until about 10 days of age, when we added brine shrimp to their diet. We began to replace APR with crushed flake food when fry reached about 20 days of age.

We performed all analyses of fitness trait measurements using R (R Development Core Team, 2008), and created a number of figures using the R package "lattice" (Sarkar 2008). As for the invasion experiment data analysis, we considered $p \leq 0.05$ to be significant.

Fecundity & Fertility: Experimental design

We measured fecundity as the total number of eggs produced by a female fish, and fertility as the proportion of intact eggs containing embryos that were successfully developing 24 hours after their collection. Several days before beginning trial 1, we randomly selected 10 females and 10 males of each genotype (W and T400), weighed them, and arranged the 20 pairs of fish in four types of crosses (with the letter representing the female genotype first, W = wild-type and T = transgenic): WW, WT, TW, and TT (Tab. 2). Between trials 1 and 2, we weighed the individuals used in trial 1 and rearranged them for use in trial 2, except for replacing a few fish that did not survive the experiment with individuals of the same sex and genotype. We used the same procedure to select fish for use in trials 3 and 4, in which we selected a different set of wild-type fish and transgenic fish were of the T67 line.

We collected eggs from females every morning for ten consecutive days, and placed eggs from each pair of fish in separate, labeled mesh-bottom cups in a flowing-water bath with a methylene blue solution to inhibit fungal growth on eggs. Twenty-four hours later, we counted the total number of eggs in each cup, the number of embryos that were successfully developing, and noted whether any eggs were broken due to handling.

Fecundity: Data analysis

We summed each female's total egg production during each ten-day trial. We excluded eight cases in which the female was replaced in the middle of a trial (Tab. 2). Four females failed to produce any eggs during the entire ten-day test period but were included in our analysis because they survived the entire experiment. We included eggs that were broken or infertile in the count of total egg production.

We analyzed fecundity data with an ANOVA in which ten-day egg production was dependent on the interaction between male and female genotypes. We included trial number as a blocking variable and starting weight of the female as a covariate in analyses.

Fertility: Data analysis

We calculated a ten-day overall fertility rate for each trial as overall fertility rate = (n fertile eggs) / (n intact eggs). To remove the effect of female genotype on male fertility, we only used eggs from wild-type females (Muir and Howard 2001). We

excluded data from two males who died during trial 1, and data from two males that were paired with a female who did not produce any eggs during the course of the trial (Tab. 2).

We repeated analyses with and without data from three males who otherwise met criteria for inclusion, but who had unusually low fertility rates. Two T400 transgenic males fertilized zero eggs and one wild-type male had an overall fertility rate of only 0.077. Because all three males were paired with females with relatively low egg production (fewer than 26 intact eggs during a trial, only 15% of mean egg production), these individuals had fewer opportunities to fertilize eggs.

We created a binomial generalized linear model for the overall fertility rate, including trial and male weight at the beginning of a trial as blocking variables, and dependent on male genotype. To correct for overdispersion in our data, we calculated dispersion parameters for each model. Because of relatively small sample sizes, we ran bootstrap simulations (n=100 simulations) and generated 95% confidence intervals (CI), with a Bonferroni correction for multiple comparisons, for the differences in fertility between male genotypes.

Mating advantage: Experimental methods

We tested the relative mating advantage of transgenic and wild-type male medaka by placing one transgenic male and one wild-type male in an aquarium with one wild-type female and scoring males according to which male successfully obtained a mating with the female. We conducted tests in five aquaria with removable dividers that separated the males from the female between mating opportunities. The only structure in

the aquaria was a small sponge filter that provided biofiltration and aeration to the aquaria. We designed the mating advantage experiments so that each combination of one female and two male fish was unique. Each wild-type female resided in the same aquarium during the entire experiment. Each of the five wild-type and five transgenic males were moved among aquaria according to a Latin square design so that any given wild-type male only competed once with each transgenic male, and all males encountered each female exactly once. We randomly selected fish for this experiment, and, before the experiment, we weighed, photographed, and noted any distinguishing features of the males so we could identify individuals during the experiment. We tested both transgenic lines for five test periods, each four to six days long, with several days in between each test period to move fish and to allow them to acclimate to their new aquaria.

During each day of a mating advantage experiment, we removed the tank dividers just before the lights turned on, and then watched the fish in all five tanks as males competed to mate with the female. We gave males a score of 0 (failure to mate with the female) or 1 (successful mating with the female) for each day of the test period. It is not uncommon to observe "trio" matings in medaka, in which both males mate simultaneously with the female (Howard et al. 2004). We noted such occurrences separately. If neither male mated with the female within approximately two hours of the beginning of observations, both males received a 0 score for that day.

We tested each transgenic line with separate sets of wild-type fish. We tested T400 males first. We randomly selected five W females, five W males, and five T400 transgenic males and assigned each to a tank. Of the five test periods with T400 males,

three lasted five days and two were extended to six days of observation due to low mating success at the beginning of the test periods. We replaced a number of W and T400 males that sickened or died between test periods, resulting in a total of 12 different T400 males and 13 different W males during the T400 experiments. We weighed fish before they were included in the experiment, but did not weigh individuals between each test period.

We tested the T67 transgenic line several months later, in five test periods that lasted exactly four days each. We weighed each fish before the experiment and between each test period. All other methods were the same as those described above for the T400 transgenic line. We replaced one W female, two W males, and two T67 males that died during the experiment, but no fish died during a test period.

Mating advantage: Data analysis

In our analysis, we excluded data from the following tanks with no successful matings: in the T400 experiments, one tank in the second test period and one tank in the fifth test period, and one tank in the fifth test period of the T67 experiments. We also excluded data from three T400 test periods during which females died and were replaced.

We used binomial generalized linear models in which wild-type matings were "successes," transgenic matings were "failures," and trio matings were excluded, and which were fitted with only an intercept term to test the odds of wild-type matings versus transgenic matings. To test the mating advantage of each genotype under the extreme scenario that the male of one genotype fertilized all eggs in trio matings, we created a second set of binomial models in which trio matings were combined as "successes" with

each male genotype in turn. We designed the rest of our analysis to predict the likelihood of one of three different types of matings during a test period: matings obtained by transgenic males alone, wild-type males alone, and trio matings.

Age at Sexual Maturity: Experimental methods and analysis

We measured age at sexual maturity by the age in days of the first five female offspring from each cross to produce viable eggs. Every morning, we visually inspected each aquarium for females with eggs. When a female with eggs was discovered, we removed her from the aquarium and collected a small sample of caudal fin tissue for PCR identification of the female's genotype. We held the first clutch of eggs from each aquarium in a flow-through egg incubator, and checked for successful development after 24 hours to ensure that at least one male in the aquarium was also sexually mature. We removed the data for one fish that we were unable to successfully PCR.

We used an ANOVA model to predict age at sexual maturity with the genotype of the sexually mature fish, and the interaction between maternal and paternal genotypes. We included trial as a blocking factor.

Juvenile Viability: Experimental methods and analysis

We measured juvenile viability by counting fish that died before reaching sexual maturity. However, we did not observe any mortalities in the aquaria before sexual maturity in trials 1 and 2. Therefore, we counted the remaining fish in trial 1 and 2 aquaria after those fish were sexually mature (at 74 and 64 days of age, respectively). For

offspring from trials 3 and 4, we closely monitored tanks for mortalities until fish reached sexual maturity. We only collected dead fry for PCR analysis in trial 3 tanks; we did not detect any mortalities before sexual maturity in trial 4.

We compared juvenile viability data for trial 1 on the basis of parental cross using a chi-squared contingency table. We excluded data for trial 2 from analysis because the number of fish in each tank after sexual maturity was greater than the number expected (*i.e.*, errors may have been made when initially stocking fry). We analyzed juvenile viability data for trial 3 with a chi-squared table and an ANOVA model where longevity in days depended on the fish's genotype and on the interaction between maternal and paternal genotypes. In our analysis for trial 3, we removed the data for two fish that we were unable to successfully PCR.

Longevity: Experimental methods and analysis

We measured longevity as the number of days fish survived. We used the same tanks of fish that we used to measure age at sexual maturity and juvenile viability. We monitored these tanks daily for mortalities, and we used PCR to identify whether deceased fish were transgenic.

We used a square-root transformation of longevity to improve normality in the data. We used an ANOVA model in which longevity depended on the deceased fish's genotype and on the interaction between maternal and paternal genotypes, with trial included as a blocking factor. We removed data from 163 fish (of a total n = 531) whose

genotype we were unable to determine using post-mortem PCR. Tukey's HSD multiple comparisons tests were used to compare the effects of different factors on longevity.

Table 1. Design of invasion experiments.

	Control	**Transgenic fish "invasion"**	
	wild-type fish (W) only (3 replicates)	T67 transgenic fish (3 replicates)	T400 transgenic fish (3 replicates)
Invasion experiment I (6 months)	W population (n = 353)	W (n = 353) + T67 (n = 20)	W (n = 353) + T400 (n = 20)
Invasion experiment II (19 months)	W population (n = 477)	W (n = 477) + T67 (n = 40)	W (n = 477) + T400 (n = 40)

Table 2. Experimental crosses for fecundity and fertility measurements; *F* = females and *M* = males. Offspring of these fish were used to measure age at sexual maturity, juvenile viability, and longevity. Only data from crosses with wild-type females were included in fertility.

Aquarium	Trial 1 (T = T400 line)		Trial 2 (T = T400 line)		Trial 3 (T = T67 line)		Trial 4 (T = T67 line)	
	F	*M*	*F*	*M*	*F*	*M*	*F*	*M*
1	T1	T1	T6	T6	T11	T11	T16	T16
2	T2	T2	T7	T7	T12	T12	T17	T17
3	T3	T3	T8	T8	T13	T13	T18	T18
4	T4[1]	T4	T9[2]	T9	T14	T14	T19	T19
5	T5[2]	T5[2]	T10[3]	T10	T15	T15	T20	T20
6	T6	W6	T1[2,3]	W1	T16	W16	T11	W11
7	T7	W7	T2	W2	T17	W17	T12	W12
8	T8	W8	T3	W3	T18[1]	W18	T13	W13
9	T9	W9	T4[2]	W4[3]	T19	W19	T14	W14
10	T10[2]	W10	T5[3]	W5[2,3]	T20	W20	T15	W15
11	W1	W1	W6	W6	W11	W11	W16	W16
12	W2	W2	W7[2,3]	W7	W12	W12	W17	W17
13	W3	W3[2]	W8	W8	W13	W13	W18	W18
14	W4	W4[2]	W9[2]	W9	W14	W14	W19	W19
15	W5	W5	W10	W10	W15	W15	W20	W20
16	W6	T6	W1	T1	W16	T16	W11	T11
17	W7[1]	T7	W2	T2	W17	T17	W12	T12
18	W8	T8	W3	T3	W18	T18	W13	T13
19	W9	T9	W4[1]	T4	W19	T19	W14	T14
20	W10	T10	W5	T5	W20	T20	W15	T15

Table 2, continued: Footnotes

[1] Female produced no eggs over the course of a trial (data included in fecundity but not fertility).

[2] Fish died and was replaced in the middle of a trial.

[3] Fish died and was replaced between trials.

Table 3. Bootstrap 95% confidence intervals (with Bonferroni correction) for male fertility by genotype. Bold numbers indicate significant differences.

Male genotype comparison	Lower bound	Upper bound
wild-type - T67	-3.295	0.908
T67 - T400	-4.428	0.437
wild-type - T400	**-6.595**	**-0.280**

Table 4. Summary of fitness trait measurement experiments; different letters in superscript indicate significant ($p < 0.05$) differences between individuals of a given genotype or in a given trial, according to tests as described in Methods.

Fitness trait	**Genotype rank**
Fecundity	$T67^{a} > W^{a} > T400^{a}$
Fertility	$T400^{a} > T67^{a,b} > W^{b}$
Mating advantage	$W^{a} > T400^{a}$ $W^{b} > T67^{c}$
Age at sexual maturity	$T67^{a} > T400^{a} > W^{a}$
Juvenile viability	$W^{a} > T67^{a}$
Longevity	$W^{a} > T67^{a,b} > T400^{b}$ maternal genotype also significant: $W^{a} > T67^{b} > T400^{b}$

Figure 1. Confirmation of transgene expression in T67 juveniles and adults and in a T400 adult; RT-PCR run on a 2% agarose gel (photo courtesy Dr. Sushmita Singh, BioMedical Genomics Center, University of Minnesota).

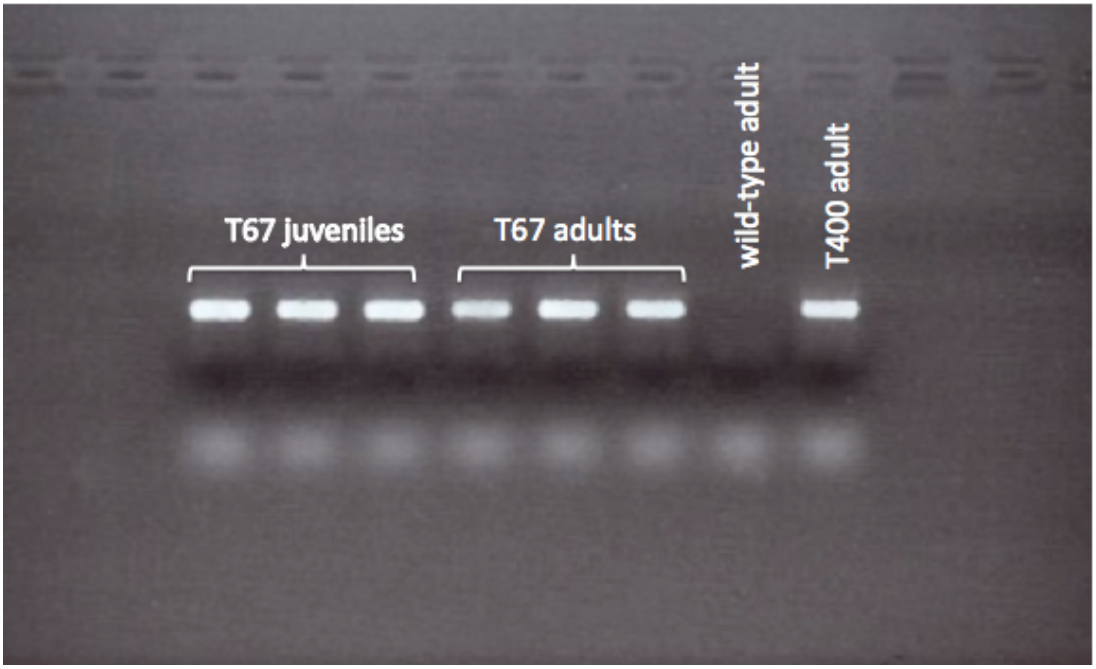


Figure 2. Initial (horizontal lines) and final (gray columns with data values on top) transgene frequencies in invasion experiments I (a) and II (b).

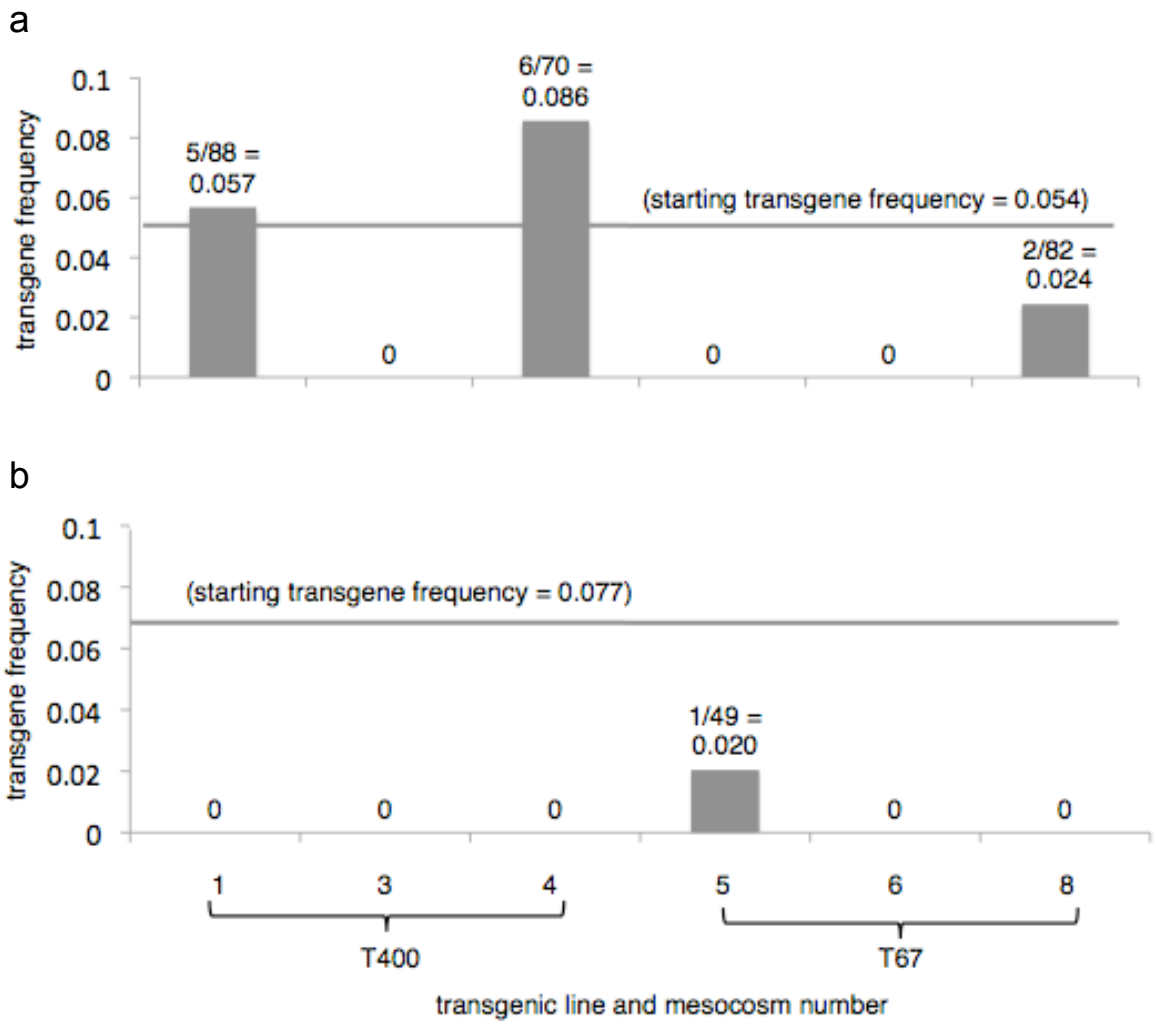
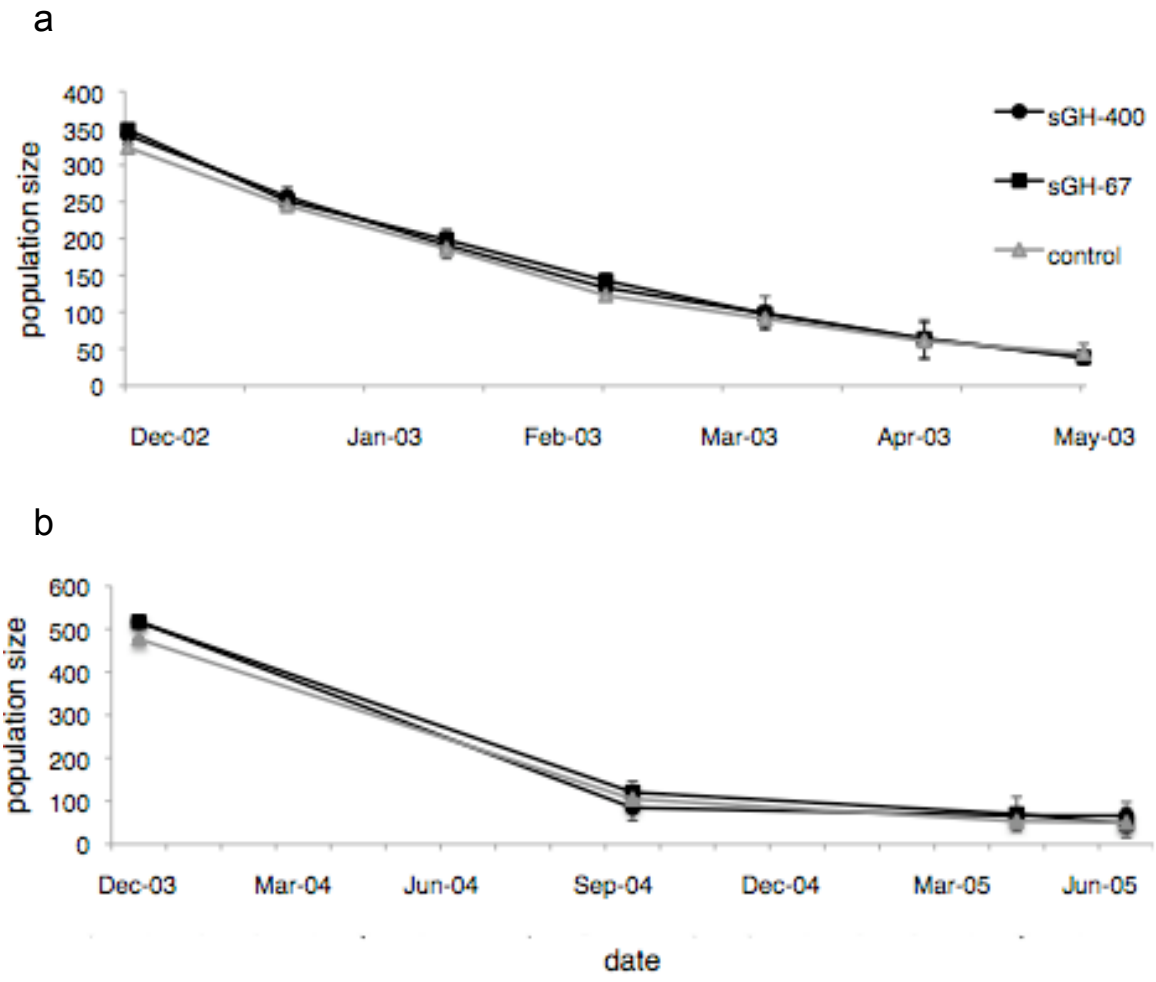


Figure 3. (a) Declining population in invasion experiment I. Population sizes are estimates based on (original population size - mortalities to date), except May 2003 measurement, which is a census size. (b) Population size during invasion experiment II. Population sizes were all determined via census. Error bars are +/-1 SD.



CHAPTER 3

Predation and food limitation influence fitness traits of growth-enhanced transgenic and wild-type fish

with Anne R. Kapuscinski

Summary

Genetically engineered fish are nearing commercialization for aquaculture production around the world. In many conventional aquaculture systems, escapes of large numbers of farmed fish are common. If transgenic fish were to escape from farms and encounter wild fish of the same species, they could interbreed, leading to transgene introgression. Relative advantages of transgenic fish for fitness-related traits could drive population processes like gene flow, but measurements of fitness traits will be most useful to risk assessments if they are taken under environmental conditions relevant to those encountered in the wild. We compared the following fitness traits of wild-type (W) and growth-enhanced transgenic (T) Japanese medaka (*Oryzias latipes*): fecundity, fertility, survival to sexual maturity, age at sexual maturity, and mating advantage. Because food availability and predation have been shown to alter the life histories of growth-enhanced transgenic fish, we measured traits under four different environmental conditions: (A) high food availability, predation absent; (B) high food availability, predation present; (C) low food availability, predation absent; and (D) low food availability, predation present. We found that across all environments, T females were more fecund than W females, and fecundity was highest in Environment B. There was a genotype × environment interaction for male fertility. Offspring of heterogeneous (TW or WT) crosses had higher survival to sexual maturity than offspring of two W parents. Fish in Environment A reached sexual maturity significantly sooner than fish in all other environments. W males obtained more matings than T males in Environments B and C. Our results suggest that risk assessment of gene flow from transgenic to wild relatives

must incorporate ecosystem-specific variability in limiting environmental factors and their potential impact on fitness components of different genotypes.

Introduction

A number of varieties of genetically engineered fish are being developed for aquaculture production around the world (Nam et al. 2007). In many conventional aquaculture systems, escapes of large numbers of farmed fish are common (National Research Council 2004). If transgenic fish were to escape from farms and encounter wild fish of the same species, these fish could interbreed, leading to transgene introgression, or transgene flow to the wild population. The need to assess the potential impacts of genetically engineered organisms on biodiversity was recently identified among the top research priorities for Conservation Biology (Sutherland et al. 2009). Assessing the risk of gene flow from transgenic fish to wild populations is a particular challenge to this kind of research (Devlin et al. 2006; Kapuscinski et al. 2007; National Research Council 2008).

Measuring fitness trait values for transgenic and wild-type fish is one way to predict the consequences of gene flow from transgenic fish into a wild population. Laboratory studies have found differences in fitness related traits of transgenic and wild-type fish, such as a mating advantage among growth-enhanced transgenic male fish (Howard et al. 2004). However, it is important to take such measurements in an environment that is relevant to the conditions that fish might encounter in the wild (Devlin et al. 2007). Previous experiments comparing the fitness trait values of wild-type

and transgenic fish have been carried out under relatively sterile laboratory conditions (Muir and Howard 1999, 2001; Howard et al. 2004). It is likely that fitness phenotypes would be affected by limiting factors in the environment (Stearns 1989; Devlin et al. 2006), but research to indicate how the relative fitness of transgenic fish might be altered by different environments is lacking. Predation and food availability are two environmental variables that have demonstrated effects on the fitness of growth-enhanced transgenic fish.

Predation has been shown to drive changes in fitness-related traits of prey species. Killifish (*Rivulus hartii*) had lower fecundity and suppressed adult growth rate in streams invaded by the piscivorous fish *Hoplias malabaricus* (Fraser and Gilliam 1992). Longnose killifish (*Fundulus majalis*) experienced lower growth rates when they were exposed to the predator sand seatrout (*Cynoscion arenarius*) (Woodley and Peterson 2003). Even chemical cues from brook trout (*Salvelinus fontinalis*) caused mayflies (*Baetis bicaudatus*) to exhibit reduced fecundity but earlier maturation at a smaller size (Peckarsky et al. 2002). Growth-enhanced transgenic channel catfish (*Ictalurus punctatus*) were less able to avoid predators compared to wild-type conspecifics (Dunham et al., 1999).

Fish are capable of expressing flexible growth in response to food availability (Sebens, 1987; Jobling, 1995). Growth-enhanced transgenic fish may have different phenotypic and behavioral responses to food availability, because of their higher feeding motivation compared to wild-type fish (Devlin et al. 1999). Compared with wild-type siblings, growth-enhanced transgenic coho salmon (*Oncorhynchus kisutch*) more readily

attacked prey items and were more likely to make repeated strikes (Sundström et al. 2004a). When subjected to low food availability, populations of coho salmon with growth-enhanced transgenic salmon among them experienced population crashes or extinctions (Devlin et al. 2004b).

The combined effects of food limitation and predation are especially interesting because prey species must choose between foraging for food and increasing their risk of predation. This trade-off has been demonstrated theoretically (Houston et al. 1993; Lima 1998) and experimentally (Werner et al. 1983; Werner and Hall 1988). The addition of growth hormone in fish tends to elevate metabolic demands (e.g., Jönsson et al. 1996), increasing the motivation of growth-enhanced fish to forage in spite of predation risk. Juvenile rainbow trout (*Oncorhynchus mykiss*) injected with growth hormone were more willing to eat in the presence of a model heron predator (Jönsson et al. 1996). Growth-enhanced transgenic coho salmon were more susceptible to predation than wild-type coho salmon, especially when food was limiting (Sundström et al. 2004b).

We measured fitness trait values of growth-enhanced transgenic and wild-type fish in environments with different levels of food availability and simulated predation. We compared the fecundity, fertility, survival to maturity, age at sexual maturity, and mating advantage of each genotype across the environments.

Methods

Model population

Japanese medaka (*Oryzias latipes*; hereafter medaka) are small fish native to a variety of aquatic environments in Southeast Asia (Hirshfield 1980). Medaka begin to reproduce around two months of age, and can mate daily for an entire spawning season (Uematsu 1990; Grant et al. 1995b). Mating activity and spawning usually takes place shortly after dawn, or in captivity, within about an hour of the beginning of the photoperiod (Hirshfield 1980; Wittbrodt et al. 2002; Shima and Mitani 2004). Partly because medaka can survive and reproduce readily under laboratory conditions (Briggs and Egami 1959; Yamamoto 1975), they are a common model species in vertebrate biology. We used a line of growth-enhanced transgenic medaka (MtsGH-67, hereafter T; produced by Dr. William Muir, Purdue University) derived from the Purdue orange-red strain, and engineered with an all-salmonid gene construct (Devlin et al. 1994) consisting of a rainbow trout metallothionein-B promoter (Chan and Devlin 1993) and sockeye salmon (*Oncorhynchus nerka*) full-length, type-1 growth hormone gene (Devlin 1993). We also used a line of wild-type (hereafter W) medaka descended from wild fish captured in Japan (obtained from Pacific Aquatics, Chatsworth, CA, USA). We confirmed expression of the transgene with RT-PCR and tested for inheritance of the transgene among offspring with PCR.

Environmental treatments: Food availability and predation

We tested two environmental factors, food availability and predation, in a completely crossed design, resulting in four environmental treatments (Table 1). Environment A is most similar to a standard laboratory, or culture, environment in which fish are well-fed and free from natural predators. At the other extreme, Environment D is most similar to a natural environment, in which food availability is lower and a risk of predation often exists. We randomly assigned each of 24 experimental units to one of these four treatments, and each unit remained assigned to the same environmental treatment for the duration of all experiments (Table 2). We then assigned each aquarium to a genotype treatment; each male-female genotype combination is represented within each of the four environmental treatments at least once, and each genotype combination is repeated exactly six times over all 24 aquaria. All 24 aquaria were used to measure survival to maturity and age at sexual maturity. Fertility, fecundity, and mating advantage experiments used a subset of the aquaria to balance the genotype and environment treatments.

We calculated feeding rates for high- and low-food availability treatments using the mass and number of fish in each aquarium. We fed fish assigned to the high-food treatment 10% of their total mass in flake food (O.S.I.) each day, divided evenly between two feedings separated by at least one, but not more than eight, hour(s). This feeding rate was essentially feeding to excess, but limited the amount of uneaten food. We fed fish in the low-food treatment at a rate of 3% of their total mass per day, delivered once daily. This feeding rate is intermediate between the two lowest feeding rates used to feed

medaka in a previous study (Hirshfield 1980). Our preliminary experiments indicated that it was sufficient to maintain fish in reproductive condition, a necessity for most of our experiments. We also fed a suspension of 24-hour-old live brine shrimp larvae (*Artemia* spp.) to high-food-availability treatments at a rate of one drop per adult fish in the aquarium, while fish in the low-food-availability treatments received one drop of brine shrimp per two adult fish.

We simulated predation with the head of a great blue heron (*Ardea herodias*) decoy (Sport Plast Decoy Company, Italy). Wading birds are a common predator of small fishes in the rice paddy environments that medaka inhabit (Maeda 2001; Iguchi and Kitano 2008). We exposed fish in predation-present treatments to the simulated predator concurrently with feeding flake food, in the front half of the tank (Figure 1). For five minutes, approximately the time it takes for the fish to eat most of the flake food and before the food begins to sink to the bottom of the tank, we held the heron's head just above the water, striking the head below the water's surface 10-20 times during a simulation. All aquaria were covered with an opaque fabric on the front, preventing fish from seeing the heron's operator. We operated the predator with movements to mimic the "stand-and-stalk" hunting behavior of a heron (Kushlan, 1976), similar to the motion used by Jönsson et al. (1996).

Experimental environment

We used 24 113.5-L glass aquaria connected by a hybrid recirculating system, with filtered well water flowing in at a replacement rate of approximately 20% system

volume per day. We contained all aquaria inside an insulated, photoperiod-controlled chamber. We maintained a photoperiod of 16:8 (l:d) and water temperature of 26°C to promote mating behavior (Shima and Mitani 2004). We separated each aquarium latitudinally into a front and back section with a Styrofoam tank divider that interrupted the water's surface (Figure 1). We provided a fry refugium consisting of a floating mesh basket with artificial spawning grass in the back half of each aquarium.

Data analysis: general

We carried out all analyses using the R statistical package (R Development Core Team 2008). We used linear models for continuous response variables, and generalized linear binomial models for responses that could be characterized as success/failure. We included blocking factors, such as trial, where applicable. We tested for significance of genotype, environmental treatment, and genotype × environment interaction terms. We applied Tukey HSD post-hoc multiple comparisons tests to investigate differences in means between genotype or environment treatments (using the multcomp package to apply Tukey tests to binomial models; Hothorn et al. 2008). We produced statistical graphs using R (including error bars coded with the package gplots; Warnes 2009). We report mean ± standard deviation for a number of traits. Where we obtained p-values, we considered values of $p < 0.05$ to be significant.

Fecundity and Fertility: Experimental design

We measured fecundity and fertility daily during four trials of ten days each, separated by an acclimation period of at least ten days. We randomly selected eight 10-12 month old medaka of each sex and genotype for these experiments. We arranged one male and one female fish in each of 16 experimental aquaria in homogeneous and heterogeneous crosses according to each tank's assigned genotype treatments (Table 2), and no single fish was paired with the same mate more than once during all trials. We exposed fish in predation-present treatments to the simulated predator twice during each ten-day test period. We weighed all fish before each trial began, and we assumed that weight remained constant during the trial to avoid additional handling. If a fish died in the middle of an experimental trial, we replaced it with a fish of the same sex and genotype, and noted the weight of the replacement fish. We collected and counted eggs each day, then maintained those eggs in a flowing-water incubator for 24 hours. The next day, we counted 24-hour-old eggs that were successfully developing under a dissecting scope.

Fecundity: Data analysis

We analyzed fecundity using linear models where the response was the total number of eggs produced by a female during a ten-day trial. Trial was retained in all models as a blocking factor, but we did not include female weight as a covariate because it was significantly and positively correlated with female genotype. We tested the importance of female genotype, environmental treatment, and genotype × environment

interaction, using Type II ANOVA to account for unbalance in our data. We excluded data from females that died during a trial.

Fertility: Data analysis

We analyzed fertility with binomial generalized linear models where fertile eggs were "successes" and infertile but intact eggs, "failures." We retained trial in all models as a blocking factor. We used F-tests to compare models with and without terms of interest: male genotype, environmental treatment, and genotype × environment interaction.

We removed data from analysis if the female produced no eggs over a 10-day trial or males were replaced in the middle of a trial. We excluded one additional datum with unusually low fecundity and fertility that was identified as an outlier by model checking procedures.

Survival to maturity and age at sexual maturity: Experimental design

We measured survival to maturity and age at sexual maturity using medaka fry. To produce offspring for these measurements, we randomly assigned a pair of six-month old medaka to each of the 24 test aquaria, according to the assigned female-male genotype combinations (Table 2). We allowed these fish to acclimate to their environments for ten days, during which fish in simulated predation treatments were exposed to the predator two times. After the acclimation period, we collected eggs from all females and placed each clutch of eggs in a recirculating-water incubator (~27° C)

with 3 ppm Methylene blue (Kordon, LLC, Hayward, CA, USA) to discourage fungal growth. We collected eggs from all tanks on the first two days after the acclimation period was complete, and for two days thereafter we continued to collect eggs from several tanks that were producing small numbers of eggs. We counted the number of fertile eggs 24 hours after the eggs were placed in the incubator.

When fry hatched, we counted them, and transferred the fry to 10 cm diameter mesh-bottom PVC baskets suspended inside all 24 aquaria. We fed dry, powdered Artifical Plankton Rotifer (APR; O.S.I. Marine Lab) twice daily in excess to all aquaria. Ten days-post-hatch (DPH), we equalized the number of fry in each basket to 30 and released those randomly selected fry into the aquarium. We introduced different foods and began to feed low and high food availability aquaria at different rates after 10 DPH. From 10 to 27 DPH, we fed 0.033 g APR suspended in water twice daily to low-food availability aquaria. We fed brine shrimp larvae to low food availability aquaria as follows: one drop from 10 DPH to 36 DPH; two drops from 37 DPH to 63 DPH; and three drops after 64 DPH. At 21 DPH we began to feed 1.5 g per day of finely-ground flake food (O.S.I.) to low food availability aquaria. For aquaria in the high-food treatment, we fed twice as much of all types of food during the aforementioned periods. We censused fry on 20, 33, 48, and 64 DPH. Beginning with the 33-DPH census we also weighed the total mass of all fish in each tank and adjusted flake food feeding rates accordingly. Between 30 DPH and the onset of sexual maturity, we simulated predation in predation-present aquaria five times.

Beginning on 43 DPH, we searched for females bearing eggs. When we observed a sexually mature female, we removed the fish from her aquarium, removed and counted her first clutch of eggs, and weighed the fish. To determine the genotype of mature females with at least one transgenic parent, we took a small sample of caudal fin tissue and analyzed the tissue with PCR. We held the eggs from the first three females to mature in each aquarium for 24 hours, and checked the eggs for fertility to confirm that males in the aquarium were also sexually mature.

Survival to maturity and age at sexual maturity: Data analysis

Survival to sexual maturity was measured in two stages: the number of fertile eggs that hatched (hatchability) and the number of fry that survived from 10 DPH to maturity (juvenile viability). Because fry were too small to genotype before sexual maturity without incurring high mortality, we used their parental genotypes as explanatory variables in models for hatchability and juvenile viability. We used binomial generalized linear models to compare the number of eggs that successfully hatched to the number of fertile eggs that did not hatch from different parental genotypes and in different environments. We also analyzed juvenile viability data using binomial models, in which fish surviving from 10 to 48 DPH were successes and fry that did not survive were considered failures. To test the effect of parental genotype and environment on overall survival to maturity, we created a linear model for the product of proportion of fertile eggs hatched × proportion of fry surviving to sexual maturity. We transformed the

response with an arc-sin square-root transformation to improve normality and tested genotype, environment, and interaction terms using Type II ANOVA.

We analyzed age at sexual maturity using linear models. To maximize balance in our data, we included only the first through ninth females to mature in each tank. We transformed the number of days to sexual maturity with a natural log to satisfy normality assumptions. Weight was excluded from age at sexual maturity models because it was highly correlated with environmental treatment. We used Type II ANOVA to compare models in which genotype of the sexually mature fish, environmental treatment, and the genotype × environment interaction were explanatory variables.

We excluded data from six fish that PCR failed to identify as T or W from the age at sexual maturity analysis. We also excluded one fish that was the only female to reach sexual maturity in aquarium 21. We had to exclude aquarium 17 from survival to sexual maturity and age at sexual maturity analyses because the adult pair of fish in tank 17 produced no offspring.

Mating advantage: Experimental design

We measured mating advantage by observing the number of successful matings obtained with a W female by a W male when competing with a T male. We housed fish in groups separated by genotype and sex (W females, W males, and T males) and each environmental treatment (i.e., 12 holding tanks total) and allowed them to acclimate to the environmental conditions for ten days before the start of the first mating advantage trial. We simulated predation five times during the acclimation period. One day before an

experimental trial, we randomly selected four females, four W males, and four T males, one from each environment. We took photographs of the selected fish for identification, and weighed and measured standard lengths for all fish.

We observed mating advantage trials in four aquaria, each assigned to a different environmental treatment (Table 2). We carried out four trials of four days each, with three-day acclimation periods between trials. Fish were not used in more than one mating advantage trial. At night, we placed the males in separate baskets inside the aquarium to prevent mating before the observation period began in the morning. Each morning of a mating advantage observation, we arrived before the lights came on and released the males from their baskets. We observed the males competing for the female in real time, and noted which male was mating with the female when she released eggs. In our experiments and in previous studies of medaka behavior (e.g., Grant et al. 1995a; Howard et al. 2004), it is possible for both males to mate with the female at the same time, and we noted these "trio matings" separately. We also recorded the number of minutes to a successful mating.

Mating advantage: Data analysis

To test the odds of wild-type males obtaining matings versus transgenic males, we created binomial generalized linear models in which W matings were "successes" and T matings were "failures." We excluded trio matings from these analyses, and fitted only an intercept term. In addition, we produced a separate model for each environmental treatment. We created a second binomial model, for all environments, in which trio

matings were divided equally between each male genotype (W and T) in turn. We also compared the time to successful mating for different mating types.

Results

Fecundity

On average, the females in our experiment produced >20 eggs per day (mean 10-day total: 209.6 ± 128.2), with individual 10-day fecundities ranging from zero to 593. The genotype × environment interaction was not significant (Type II ANOVA: $F_{3, 46} = 1.056$, $P = 0.347$), so the interaction term was dropped from the model. Using only main effects we found that environmental treatment (Type II ANOVA: $F_{3, 49} = 6.387$, $P = 0.001$) and female genotype (Type II ANOVA: $F_{1, 49} = 5.606$, $P = 0.022$) were both significant model terms.

Ten-day egg production totals for T females were higher than for W females (W: 180.1 ± 115.1; T: 240.1 ± 135.8; Tukey HSD: adj. $P = 0.023$). Transgenic females were heavier than W females (T: 0.340 ± 0.082 g; W: 0.282 ± 0.117 g; Tukey HSD: adj. $P = 0.036$). Egg production overall was higher in Environment B than in Environments C and D (Tukey HSD: B-C adj. $P = 0.001$; B-D adj. $P = 0.026$; Figure 2). Examining only the food availability factor, high food availability was positively correlated to egg production (Tukey HSD: adj. $P < 0.001$). Predation treatment level was not correlated with egg production (Tukey HSD: adj. $P = 0.136$).

Fertility

Across all environments, male fertility in our experiments was high (Figure 3), and was not different between male genotypes (W: 0.97 ± 0.09; T: 0.96 ± 0.09). While fertility ranged from 0.62 (W) or 0.63 (T) to 1.00, most fertility rates were greater than 0.95 (among W males: 26 of 28; T males: 23 of 28).

In binomial models of fertility, the genotype × environment interaction was significant ($F_{3, 45} = 3.512$, $P = 0.023$). However, multiple comparisons tests did not reveal differences between genotype-environment combinations (Tukey HSD: all adj. $P > 0.579$). Alone, neither genotype nor environment had significant effects on fertility ($F_{1, 48} = 1.731$, $P = 0.195$; $F_{3, 48} = 0.691$, $P = 0.562$).

Survival to maturity

Across all treatments and parental genotypes, 0.90 (± 0.08) of the fertile eggs hatched, and 0.84 (± 0.16) of hatched fry survived from 10 to 48 DPH. In binomial models describing hatch proportion, the genotype × environment interaction was not significant ($F_{9, 22} = 1.121$, $P = 0.451$). Without the interaction term, neither parental genotype combination ($F_{3, 22} = 2.573$, $P = 0.090$) nor environmental treatment ($F_{3, 22} = 2.105$, $P = 0.140$) was significant.

As expected, juvenile viability decreased as environments got "worse," from 0.90 (± 0.08) in Environment A to 0.76 (± 0.14) in Environment D. In binomial models of juvenile viability, the genotype × environment interaction term was not significant ($F_{9, 22} = 0.733$, $P = 0.675$). When the interaction term was dropped, the parental genotype

combination was significant ($F_{3, 22} = 5.654$, $P = 0.008$), but the environmental treatment was not significant ($F_{3, 22} = 1.206$, $P = 0.339$). Multiple comparisons of means tests revealed that fry with parents of one T and one W genotype (i.e., TW or WT) had higher survival rates than fry with WW parents (Tukey contrasts: both adj. $P < 0.007$).

The proportion of fertile eggs hatched × the proportion of fry surviving from 10 DPH to sexual maturity attempts to capture the overall proportion of fry surviving from fertilization to maturity (Figure 4). In this linear model, the parental genotype × environment interaction was not significant (Type II ANOVA: $F_{9, 22} = 2.316$, $P = 0.140$). Without the interaction term, environmental treatment was not significant (Type II ANOVA: $F_{3, 22} = 1.713$, $P = 0.204$), but parental cross was (Type II ANOVA: $F_{3, 22} = 7.861$, $P = 0.002$). As with juvenile viability alone, the rate of survival to maturity for offspring of WT and TW crosses was greater than that of offspring from WW crosses (Tukey HSD: adj. $P = 0.001$ and 0.020, respectively).

Age at sexual maturity

We detected the first sexually mature female at 47 DPH, a T female in Environment B. The first female to reach maturity in a low-food-availability treament was a T female in Environment D, at 51 DPH. All clutches of eggs that we examined were fertile, indicating that males in all aquaria matured before or at the same time as females.

The genotype × environment interaction was not significant (Type II ANOVA: $F_{3, 177} = 0.174$, $P = 0.914$). In a model with only main effects, environment was highly

significant (Type II ANOVA: $F_{3, 180} = 35.078$, $P < 0.001$) though genotype was not (Type II ANOVA: $F_{1, 180} = 0.952$, $P = 0.331$). Fish in Environment A matured sooner than fish in all other environments (Tukey HSD: all adj. $P < 0.011$; Figure 5). Fish in Environment B matured sooner than those in Environments C or D (Tukey HSD: both adj. $P < 0.001$). Maturation time was not different between fish in Environments C and D (Tukey HSD: adj. $P = 0.599$).

Transgenic females were the same size as W females when they reached maturity (T: 0.164 ± 0.045 g; W: 0.173 ± 0.052 g; Tukey HSD: adj. $P = 0.156$). There was also no difference in the size of the first clutch produced by W or T females (T: 5.552 ± 3.495 eggs; W: 6.100 ± 4.884 eggs; Tukey HSD: adj. $P = 0.352$). However, weight and clutch size were both higher in Environment B. The first clutch size in Environment B (8.000 ± 4.609 eggs) was larger than the first clutch size in all other environments (A: 5.000 ± 2.884; C: 5.085 ± 4.318; D: 4.476 ± 2.597; Tukey HSD: all adj. $P < 0.001$). Females in Environment B (0.198 ± 0.039 g) were larger than the mature females in all other environments (A: 0.167 ± 0.029 g; C: 0.151 ± 0.051 g; D: 0.147 ± 0.050 g; Tukey HSD: all adj. $P < 0.001$). When the environmental treatment was broken into its constituent factors, both the presence of predation and high food availability had positive effects on the weight of females at sexual maturity (Tukey HSD: adj. $P = 0.018$ and $P < 0.001$, respectively).

Mating advantage

At least one male successfully mated with the female in each tank for four days during all test periods. Of this total of 64 observed matings, 36 were by W males alone, 20 were by T males alone, and 8 were trio matings in which both males participated. The T males used in our mating advantage experiments were larger on average than the W males (T: 0.420 ± 0.056; W: 0.305 ± 0.043; Tukey HSD: adj. $P < 0.001$). W and T males alone managed to copulate with the female relatively quickly (W: 10.167 ± 8.433; T: 7.150 ± 7.155 minutes) after the trial began, whereas trio matings took longer to achieve (28.500 ± 19.950 minutes; Tukey HSD: T- or W-trio, both adj. $P < 0.001$; W-T, p = 0.537).

Binomial models fitted with only an intercept term found that, across all environmental treatments, W males alone were successful more often than T males alone (intercept = 0.588, $z_{df=15} = 2.108$, $P = 0.035$). When we divided trio matings evenly between T and W males, the W advantage remained (intercept = 0.511, $z_{df=15} = 1.978$, $P = 0.048$). We created separate models for each environmental treatment, and found that W males had a mating advantage in Environments B and C (intercept = 1.792, $z_{df=3} = 2.346$, $P = 0.019$; intercept = 1.386, $z_{df=3} = 2.148$, $P = 0.032$, respectively; Figure 6). Neither male had a significant advantage in Environment D (intercept = 0.470, $z_{df=3} = 0.824$, $P = 0.410$) or Environment A (intercept = -0.916, $z_{df=3} = -1.549$, $P = 0.121$).

Discussion

Our results indicate that future risk assessments should test fish under environmental conditions relevant to those encountered in the wild. We found differences in fitness trait values for both genotypes in different environments (Table 3). Though many fitness values were greater in Environments A or B, there was no overall trend with regard to genotype: for example, T females were more fecund than W females, but W males obtained more matings than T males. Such mixed results imply that population-level processes like gene flow will be difficult to predict from measurements of one or two fitness traits taken on fish in laboratory environments. Therefore, risk assessment of transgene flow requires estimates of fitness traits that span the entire life cycle.

Fecundity

Transgenic females produced more eggs than W females over ten days, which could be related to the phenotypic effect of the transgene: T females were larger on average than W females, and larger medaka have been found to produce larger clutch sizes (Teather et al. 2000). Muir and Howard (2001) found that growth-enhanced transgenic females produced more eggs than wild-type females. In our study, though both genotypes enjoyed a positive correlation between fecundity and weight, the weight × genotype interaction was not significant (Type II ANOVA: $F_{1, 47} = 1.013$, $P = 0.319$), indicating that the effect of weight did not differ between T and W females.

The increase in fecundity in high-food treatments could be attributable to the known relationship between size and fecundity in fish (e.g., Bagenal 1978; Blueweiss et

al. 1978; Jobling 1995). Compared to female medaka in a low-food-availability aquarium, females in a high-food-availability treatment gained 0.099 g more weight on average (Tukey HSD: adj. $P < 0.001$). While our experiments were performed with only two fish per tank, a previous study of medaka competing for food found that growth and fecundity were highest among females able to gain the most access to a limited food resource (Bryant and Grant 1995).

Predation treatment alone did not result in a significant effect on the fecundity of female medaka. Though we expected fecundity to decrease in predation-present treatments based on prior research, we did not observe a negative relationship between fecundity and predation (Figure 2). Female mayflies exposed to the threat of predation had decreased fecundity relative to those in predator-free streams (Peckarsky et al. 2002). When killifish (*Rivulus hartii*) were exposed to a predation threat in an experimental stream, their total egg production decreased by about half, and more fish failed to produce eggs when exposed to a predation threat (Fraser and Gilliam 1992). Contrary to Fraser and Gilliam's (1992) results, we found that simulated predation was positively related to females producing at least one egg on any given day (binomial model: $F_{1,1322} = 17.192$, $P < 0.001$).

Fertility

The high fertility rates that we observed are comparable to previous studies of male fertility in medaka (Muir and Howard 2001; control groups in Nakayama et al. 2004). Though it was difficult to detect differences in fertility between treatments, the

genotype × environment interaction is likely related to the genotype-rank change in Environment D where W males outperformed T males (Figure 3).

Survival to maturity

Overall survival from fertile egg to maturity was dependent on parental genotype. Muir and Howard (2001) measured viability from fertile egg to three-day-old fry, and found the greatest reduction in survival during this period was among offspring of TT parents. In our combined analysis of survival to maturity, offspring from WW parents were at an even greater survival disadvantage than fish from TT parents (Figure 4): our T and W fish came from different genetic backgrounds. We may have observed higher survival among heterogeneous crosses due to the simple effect of heterosis, or "hybrid vigor" (Falconer 1989).

Age at sexual maturity

As expected, fish in Environment A (high food availability, no predation) matured soonest. The addition of simulated predation in Environment B increased the age at maturation. In the low-food environments, however, there was no difference between Environment C and D (without and with predation, respectively). The increased weight and clutch size in Environment B could be related to the delayed maturation observed in that treatment compared to Environment A. Though they had a greater opportunity to grow larger due to the high-food availability, their maturation may have been forestalled

by the threat of predation. Even though fish in Environments C and D matured an average of ten days later than fish in Environment B, they were smaller.

Predation pressure has been shown to alter the age at sexual maturity in fish. Male guppies (*P. reticulata*) in environments with predators matured at a smaller size than their counterparts in predator-free populations (Reznick and Endler 1982). In their home environments, bluegills (*Lepomis macrochirus*) in a population exposed to predation on juveniles matured later and at a larger size than those in a population where mainly adults were subject to predation (Belk 1995). However, when the bluegills from the two populations were raised together in a common garden experiment they matured at approximately the same age. Like our study, this indicates that environmental factors may have a greater effect on age at maturity than genotype.

When organisms are forced by their environment to grow more slowly, age at sexual maturity might be affected in a number of different ways. Slower-growing organisms might "1) mature later at a smaller size, 2) mature later at the same size, 3) mature later at a larger size, 4) mature earlier at a smaller size, or 5) mature at the same age at a smaller size" (Stearns and Koella 1986: p. 894). Generalizations (1), (2), and (4) are supported by research on fish reviewed in the same paper. Our results correspond most closely to generalizations (1) or (2): females in Environments C and D, presumably subject to slower growth because of their lower feeding rations, matured an average of ten days later than females in Environment B. They were smaller and produced smaller first clutch sizes than females in Environment B, but were not different in these respects from the early-maturing females in Environment A. Earlier maturation can lead to greater

fitness by increasing an organism's reproductive lifetime (e.g., Stearns 1992). Therefore, our results suggest that medaka of either genotype may express earlier ages at maturity in certain environments, and thereby gain a fitness advantage.

Mating advantage

Overall, W male medaka were most successful in obtaining matings with a W female medaka. This advantage persisted in spite of the larger size of T males compared to their W competitors. Our results contradict the common assumption that larger males tend to obtain more matings with females (e.g., Andersson 1994), and experiments that find larger male fish have a mating advantage over smaller males (e.g., Bisazza and Marconato 1988). Laboratory experiments under low-stress environmental conditions with medaka have also found that larger males (Howard et al. 1998) and larger, growth-enhanced transgenic males (Howard et al. 2004) have a mating advantage over their smaller and/or wild-type competitors. Our counter-precedent results with respect to weight and mating advantage could be due to differences in the genetic background of our W medaka (e.g., Kapuscinski et al., 2007). In our experiments, T males were derived from the Purdue orange-red strain and W fish descended from medaka from Japan; previous medaka mating advantage studies (Howard et al. 1998; Howard et al. 2004) put larger males of each genotype in competition with males derived from the same genetic background (the Purdue orange-red strain).

Though the minority of matings observed in our experiments involved both males participating in a trio mating, the fact that these matings took about three times longer

than a single-male mating indicates that competition between males may have been greatest in these cases. In fact, the weight advantage of T males in trio matings was less than the weight advantage of T males that obtained matings alone (Tukey HSD: adj. $P = 0.016$). In other words, a mating advantage trial with two males of similar size might be more likely to end with a trio mating. The likelihood of a second male joining a mating has been found to increase with female body size in fish (Marconato and Shapiro 1996). In our study, female weight was negatively related to the occurrence of trio matings, though the relationship was not significant.

W males obtained more matings than T males in two environments: one with low food availability (Environment C) and one with predation (Environment B). In fish species like the medaka in which males actively court the female, there is an energetic cost to courtship. One might then expect that the larger, T males would have an advantage in low-food availability conditions, but our results ran counter to this assumption. Female medaka can refuse the attentions of a courting male by enacting a "heads-up" display (Uematsu 1990; Grant and Green 1996). Though our experiments were not designed to test the effect of female choice, previous research on fish has found decreased female choosiness with regard to male size (Forsgren 1992) and coloration (Godin and Briggs 1996) when fish are exposed to a predator.

Neither W or T males had a significant advantage in Environments A or D. Previous mating advantage tests that have studied medaka under low-stress laboratory conditions (akin to our Environment A) have found that transgenic males dominated wild-type competitors (Howard et al. 2004); and our contradictory result may be due to

the different genetic backgrounds of our W and T fish. Our results for Environment D suggest that while a food-limited or predation-present environment might allow smaller W males to gain a mating advantage, the existence of both stresses in a single environment may be acting on both male genotypes and the females in ways that erase a clear advantage from size or genotype. Further research on relative mating advantage of T and W genotypes from different genetic backgrounds and in different environments may be warranted, as models have suggested that the risk of invasion by transgenes is very sensitive to mating advantage (Muir and Howard 1999; Aikio et al. 2008a).

Conclusion

The relative fitness trait values of growth-enhanced transgenic medaka were influenced by both genotype and environment, and not always in expected ways. Our study indicates the importance of measuring trait values under relevant environmental conditions that take into account the phenotypic effect of the transgene. Future risk assessment experiments should work to improve methods to incorporate environmental factors into confined laboratory environments. In spite of this sub-optimal state of understanding, biotechnologists and regulators are likely to forge ahead with the conduct of risk assessments to inform decisions about proposals for commercialization of transgenic fish (Aqua Bounty Technologies 2008; Food and Drug Administration 2009). Our results show that such risk assessments must incorporate ecosystem-specific variability into estimates of the likelihood and consequences of gene flow from transgenic to wild relatives. Given the data-poor context for this task, it is essential to

combine analysis of data collected on both the fish populations and ecosystems in question with quantitative methods of uncertainty analysis (Devlin et al. 2007; Hayes et al. 2007; Kapuscinski et al. 2007).

Table 1. Fitness measurements of transgenic and wild-type medaka were measured under four different environmental conditions, generated by crossing two levels of the factors food availability and simulated predation.

		Simulated predation	
		absent	*present*
Food availability	*high*	A: "laboratory" environment	B: predation
	low	C: limited food	D: "natural" environment

Table 2. Assignment of aquaria to environmental (Env.) and genotype treatments. All aquaria were used for survival to maturity and age at sexual maturity experiments. Superscripts indicate use of aquaria in other experiments.

Aquarium number	Food availability	Simulated predation	Env. treatment	Female genotype	Male genotype
1 [b]	low	absent	C	T	T
2 [a, b]	low	present	D	W	W
3 [a, b]	high	absent	A	W	W
4 [a]	high	absent	A	W	T
5 [a]	high	present	B	T	W
6 [a]	low	absent	C	W	T
7 [b]	high	present	B	T	W
8 [a]	low	present	D	T	W
9 [a, c]	low	present	D	T	T
10 [a, c]	high	present	B	T	T
11 [c]	low	absent	C	T	W
12 [a, c]	high	present	B	T	T
13 [c]	high	absent	A	W	W
14 [a, c]	high	present	B	W	W
15 [a]	low	absent	C	T	W
16 [a, c]	low	present	D	W	W
17	low	present	D	T	T
18 [c]	low	present	D	W	T
19 [c]	high	absent	A	T	T
20 [a, c]	low	absent	C	W	W
21 [a]	high	absent	A	W	T
22 [a]	high	absent	A	T	W
23 [a, c]	low	absent	C	W	T
24 [c]	high	present	B	W	T

Table 2, continued: Footnotes

[a] Aquarium used in fecundity and fertility experiments

[b] Aquarium used in mating advantage experiments

[c] Aquarium used to acclimate fish before participation in mating advantage experiments

Table 3. Summary of fitness trait measurement experiments; different letters in superscript indicate differences ($p < 0.05$) between individuals of a given genotype or in a given trial (pairwise Tukey HSD tests except for mating advantage). Genotype for survival to sexual maturity is parental genotype combination.

Fitness trait	Overall genotype effect	Overall environment effect	Genotype × environment interaction
Fecundity	$T^a > W^b$	$B^a > A^{a,b} > D^b > C^b$	$P = 0.347$
Fertility	$W^a > T^a$	$C^a > B^a > D^a > A^a$	$P = 0.023$
Survival to maturity	$WT^a > TW^{a,b} > TT^{b,c} > WW^c$	$A^a > B^a > C^a > D^a$	$P = 0.140$
Age at sexual maturity	$T^a < W^a$	$A^a < B^b < C^c < D^c$	$P = 0.914$
Mating advantage	$W^a > T^b$	*NA*	*NA*

Figure 1. Configuration of each aquarium used in experiments.

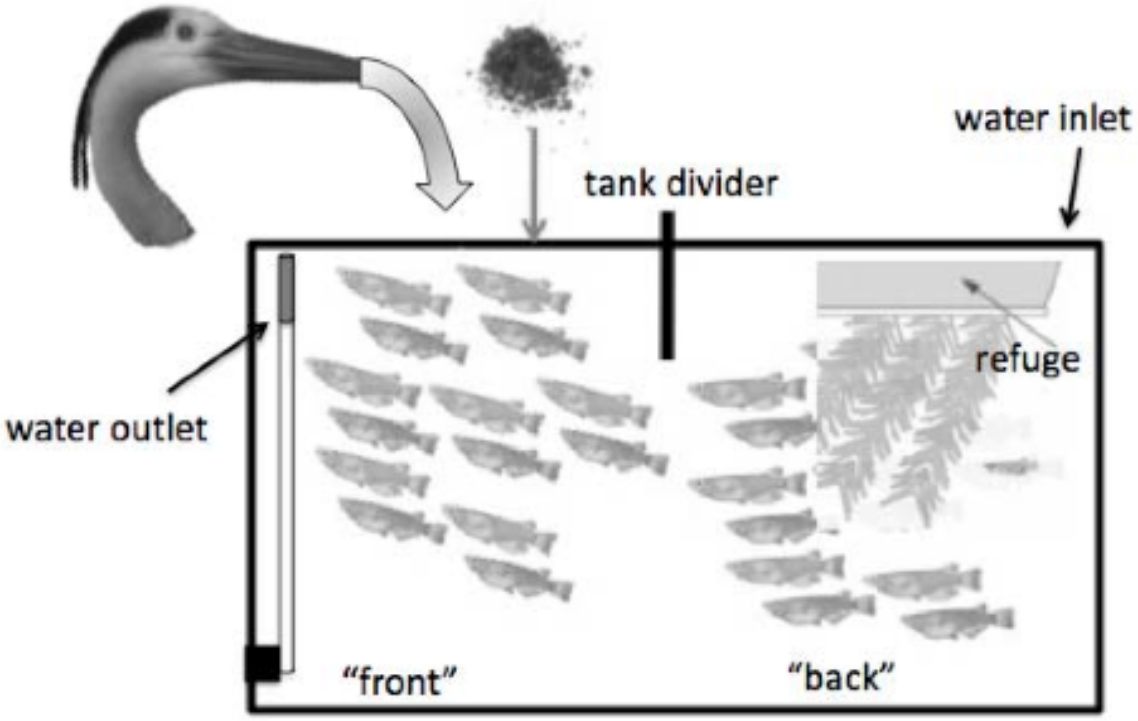


Figure 2. Mean fecundity values for T and W females in each environmental treatment. Bars represent ± one standard error.

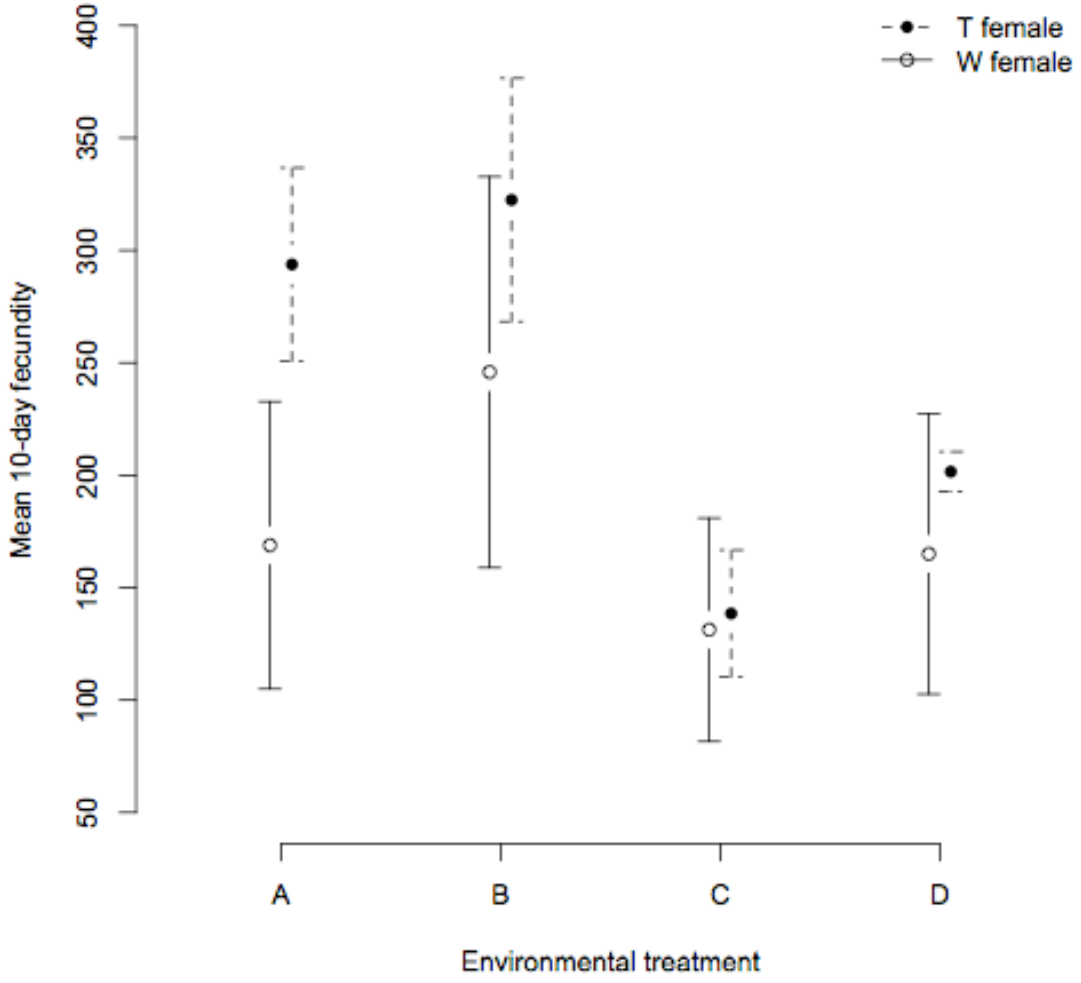


Figure 3. Mean proportion of eggs fertilized by T and W males in each environmental treatment. Bars represent ± one standard error.

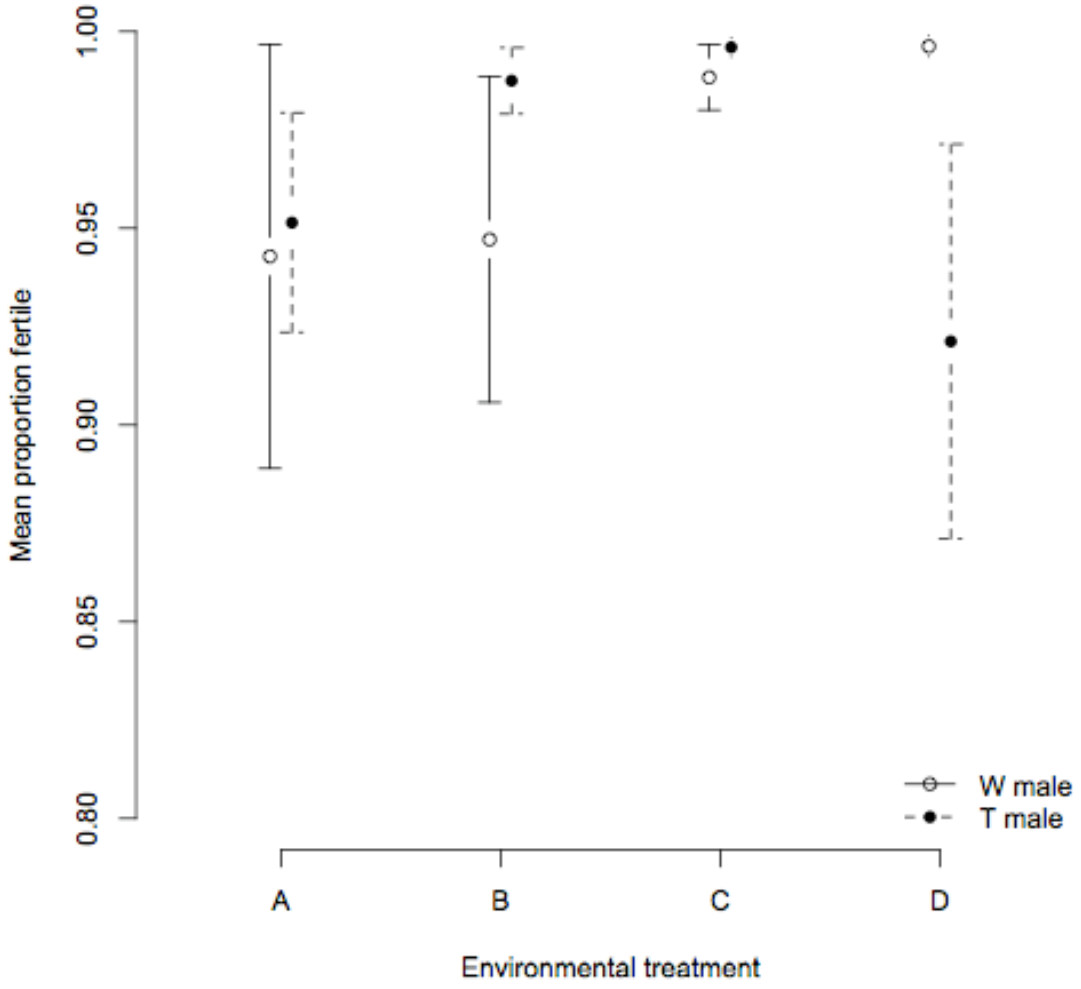


Figure 4. Proportion of fertile eggs hatched × proportion of fry surviving from 10 DPH to 48 DPH in each environmental treatment. Points are values for each parental genotype combination; lines connect the points for each parental genotype combination for ease of interpretation.

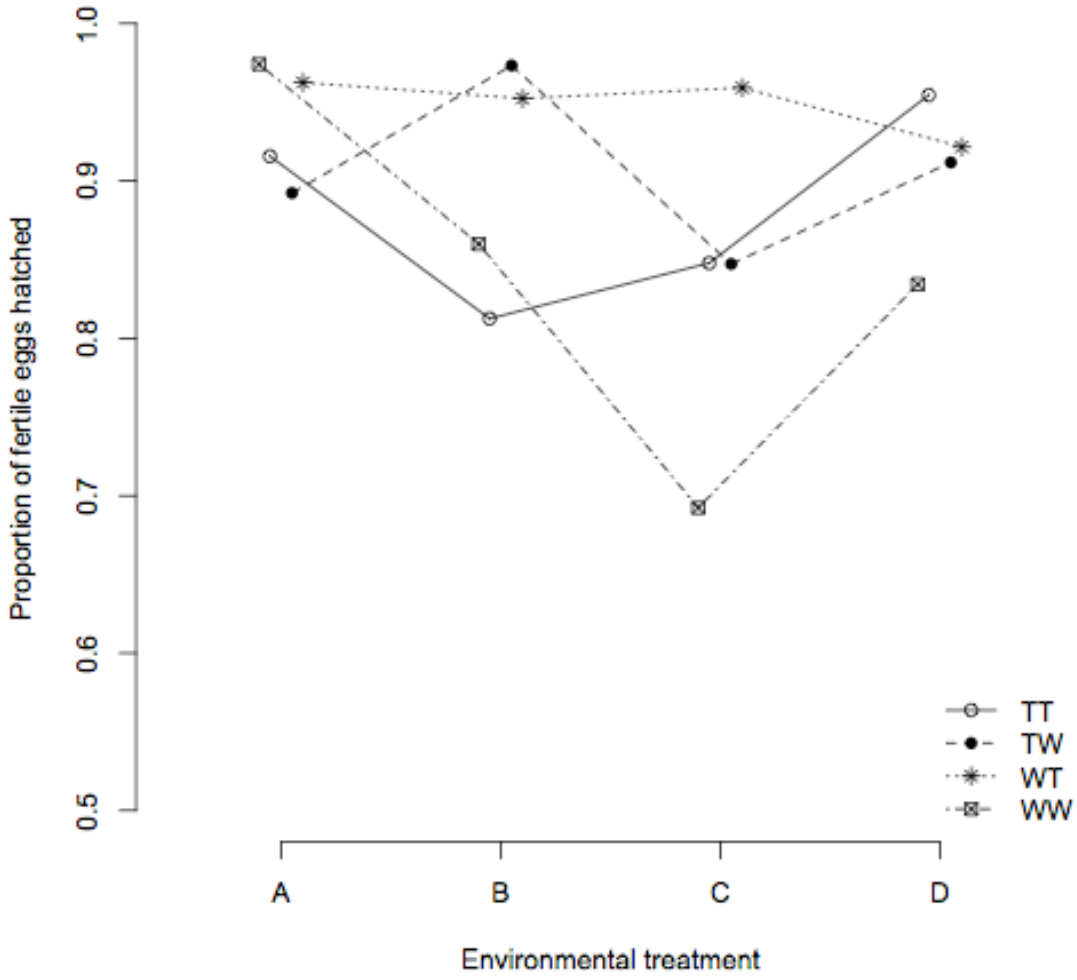


Figure 5. Mean age at sexual maturity for T and W offspring in each environmental treatment. Bars represent ± one standard error.

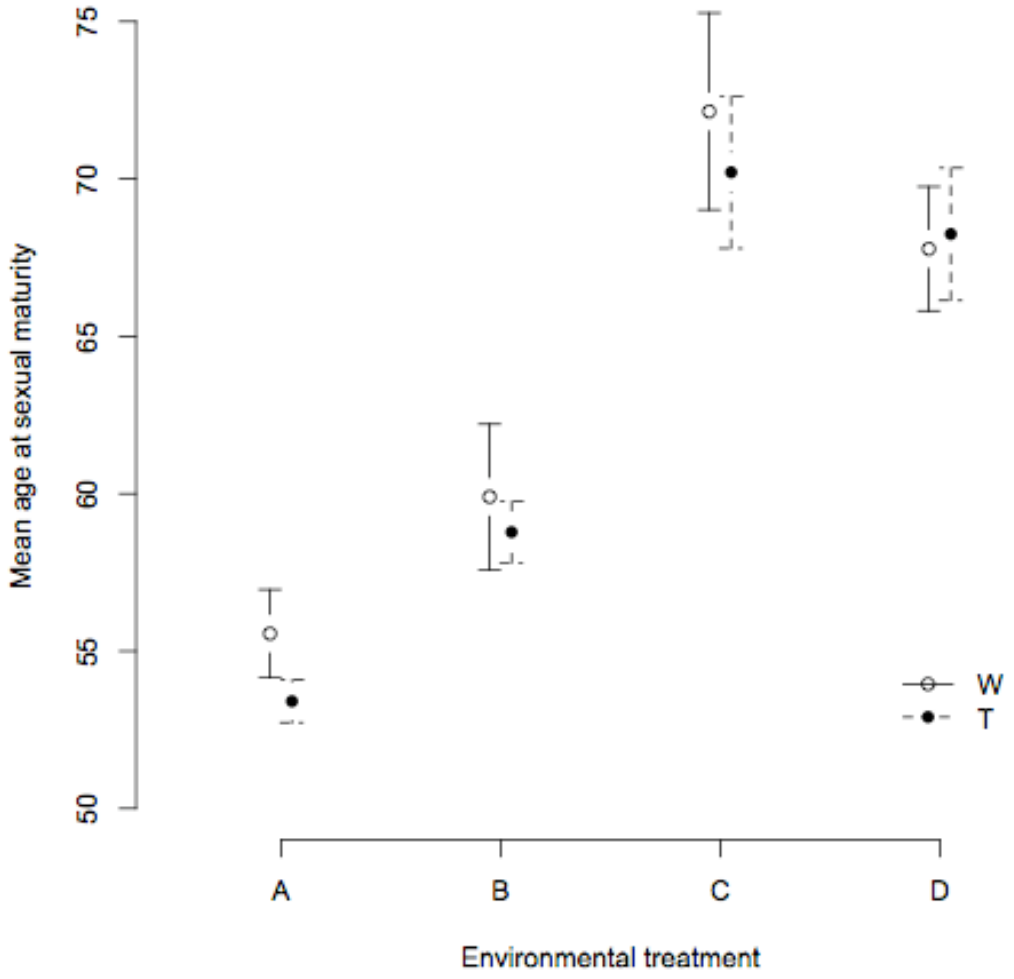
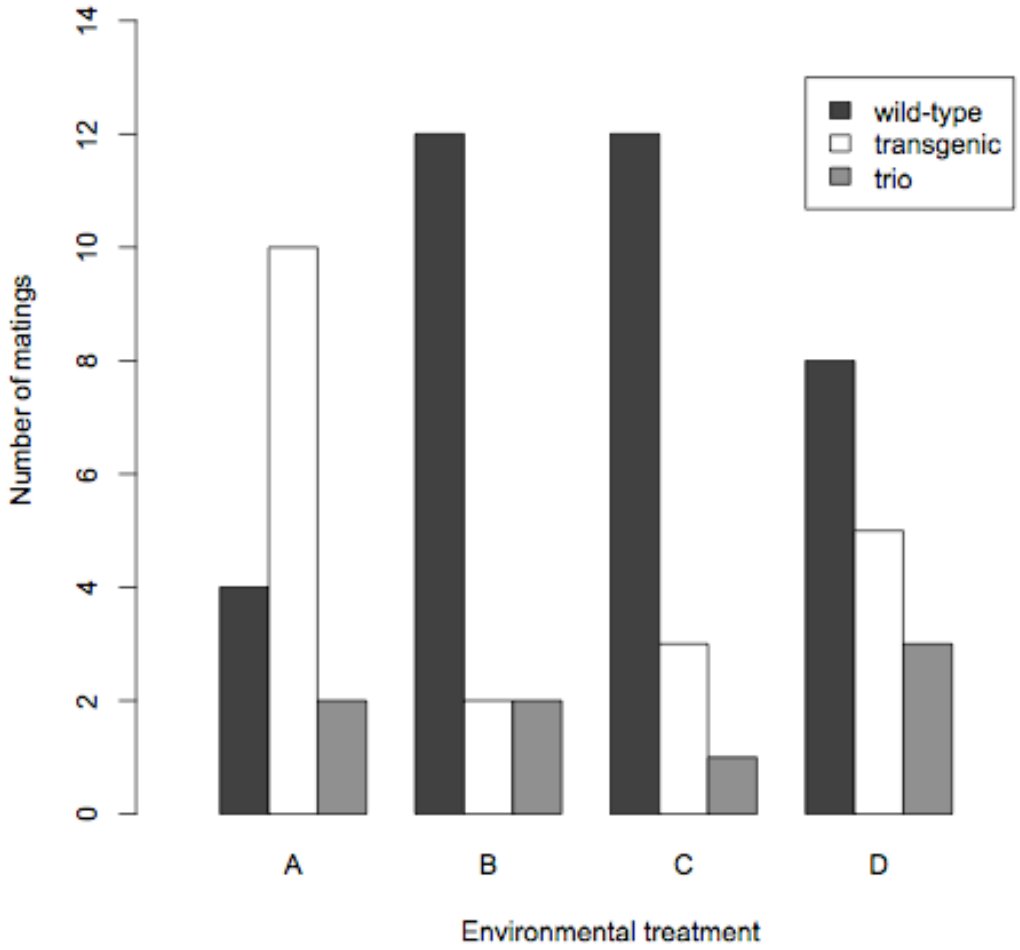


Figure 6. Occurrence of W, T, and trio matings in each environment.



CHAPTER 4

Experimental measurement and stochastic modeling of multi-generational gene flow from transgenic to wild fish under varying environments

with Anne R. Kapuscinski

Summary

Transgenic fishes are nearing commercialization for aquaculture around the world. Farmed transgenic fish would likely escape from typical production facilities and interbreed with wild relatives. Models could help risk assessors predict the likelihood and consequences of transgene flow; however, predictions from models, such as the net fitness model, have not been confirmed using real populations of transgenic fish. This requires experiments in confined environments with conditions relevant to those fish might encounter in nature. We introduced transgenic Japanese medaka *Oryzias latipes*, bearing a salmonid growth hormone gene construct, and wild-type medaka into four environments: (A) high food availability, no predation; (B) high food availability, simulated predation; (C) low food availability, no predation; and (D) low food availability, simulated predation. We maintained 24 populations of wild-type and transgenic fish under these environments for approximately three generations and measured population size and transgene frequency at 210 days. We created deterministic and stochastic versions of a demographic simulation model, parameterized with fitness trait values collected under the same environmental conditions, to predict transgene frequency under each environment, and compared observed results to model predictions. In experimental populations, final transgenic population size was greater in Environment A than in all other Environments. The final transgene frequency in Environment A (0.332) was greater than that in Environment C or D. Both models predicted that transgene frequency in Environment A would be the highest, but also overestimated transgene frequency compared to observed results. Fecundity, fertility, juvenile viability,

and mating advantage, when varied randomly over ranges observed in experiments, led to predicted transgene frequencies that overlapped with observations in Environments B and C but not in the more extreme Environments A and D. Our results illustrate the danger of measuring fitness traits in one sterile environment to parameterize a deterministic model, and use model outcomes to inform risk assessment decisions. We strongly recommend building uncertainty analysis into gene flow models, at least by incorporating parameter variability, and confirming model predictions with data collected under relevant environmental conditions before using such models to inform ecological risk assessments.

Introduction

Genetically engineered fish may pose risks to natural ecosystems, ranging from the risk of gene flow to wild conspecifics to the potential for interspecific impacts and changes to the abiotic characteristics of an ecosystem (Kapuscinski and Hallerman 1991; Kapuscinski et al. 2007; Devlin et al. 2007). Transgenic fish with a variety of traits are being developed for use in aquaculture (Zbikowska 2003; Kapuscinski 2005; Nam et al. 2007). Escaped farm fish have genetic and behavioral impacts on wild conspecifics (e.g., Fleming et al. 2000; McGinnity et al. 2003; Naylor et al. 2005), and genetically engineered farmed fish may have similar impacts. Introgression was a main contributing factor to the extinction of three fishes listed under the Endangered Species Act (Rhymer and Simberloff 1996); introgression of a transgene into a wild population could pose a novel threat to already imperiled populations (Kapuscinski et al. 2007). Transgenic fish

pose new challenges to environmental biosafety (Kapuscinski 2005), and predicting the likelihood and population consequences of gene flow is particularly challenging for ecological risk assessment (e.g., Devlin et al. 2006).

Because genetically engineered fish are difficult to contain (National Research Council 2004), experiments on gene flow from transgenic fish have been confined to laboratory environments (Chapter 2) and demographic model simulations (e.g., Muir and Howard 1999; Hedrick 2001; Muir and Howard 2001; Aikio et al. 2008a,b; Valosaari et al. 2008). The "net fitness model" has been advanced as a promising risk assessment tool (Muir 2001; Muir 2002; Muir and Howard 2002b); and its grounding in classical population genetics models (e.g., Prout 1971a,b) makes it amenable to improvement and extension to other taxa (Hallerman 2002, Muir and Howard 2002a; ISB 2004). However, the net fitness model has not yet been confirmed (Oreskes et al. 1994) with gene flow data from real transgenic fish. Although the net fitness model has been at least parameterized with data from real transgenic fish (Muir and Howard 1999; Muir and Howard 2001; Howard et al. 2004), these data were collected in relatively sterile laboratory environments.

Ecological risk assessment scientists recognize the value of introducing semi-natural environments into confined laboratory systems (Devlin et al. 2006), but only recently have such experimental designs been applied to risk assessment of transgenic fish, often by varying one or two aspects of the environment such as food availability or predation (Devlin et al. 2004b; Sundström et al. 2004b; Tymchuk et al. 2007; Sundström et al. 2009). The construction of an outdoor facility with multiple plant and animal

species for ecological risk assessment of genetically engineered fish has been reported in China (Hu et al. 2007), but no experimental results have emerged to date.

Environments with food limitation and predation have impacts on the fitness of growth-enhanced transgenic fish, because faster-growing transgenic fish may be more willing to compete for limited food resources, even under the threat of predation. Fish with higher levels of growth hormone have higher metabolic demand (Jönsson et al. 1996), and salmon engineered for faster growth have demonstrated a higher feeding motivation compared to unmodified fish (Devlin et al. 1999). Growth-enhanced transgenic coho salmon (*Oncorhynchus kisutch*) fed more aggressively, making repeated strikes and faster attacks, on novel prey items than control salmon (Sundström et al. 2004a). Increased metabolism could drive growth-enhanced fish to take more risks to obtain food items, particularly in the presence of predation. For example, after a simulated attacked from a model heron predator, young rainbow trout (*Oncorhynchus mykiss*) treated with growth hormone more readily devoured food than their un-enhanced conspecifics (Jönsson et al. 1996). Increased predation mortality compared to wild-types has been observed in growth-enhanced transgenic channel catfish (*Ictalurus punctatus*) (Dunham et al. 1999) and in coho salmon (Sundström et al. 2004b); in the latter study, predation mortality was even higher among transgenic salmon when food availability was lower. The effects of reduced food availability on transgenic fish can have population-level consequences: when food was a limiting resource, fish in laboratory populations composed of only wild-type coho salmon survived significantly better than populations composed of some or all growth-enhanced transgenic salmon (Devlin et al. 2004b).

Though these experiments show that food and predation affect transgenic and wild-type fish differently, the effect of different environments on multigenerational processes like gene flow has not been investigated. We therefore tested how two environmental variables, food availability and predation, affect gene flow in populations of growth-enhanced transgenic and wild-type Japanese medaka (*Oryzias latipes*). We also created a demographic simulation model parameterized with fitness data collected on medaka under these same environmental treatments. We ran both a deterministic model and a Monte Carlo simulation model in which selected fitness parameters were randomly varied over observed values. We compared deterministic and stochastic model predictions of transgene frequency to results observed in populations under four environments, in order to confirm the model's predictions.

Methods

We conducted the following experiments from 2008-2009 at the Aquaculture Laboratory at the University of Minnesota. We met requirements for our Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee (IBC), which set guidelines for animal welfare and confinement of genetically engineered fish, respectively.

Model population

The utility of the Japanese medaka (*Oryzias latipes*; hereafter medaka) as a model organism for vertebrate biology, genetics, developmental biology, and toxicology can

hardly be overstated and has been reviewed extensively (Myers 1952; Briggs and Egami 1959; Yamamoto 1975; Wittbrodt et al. 2002; Shima and Mitani 2004); medaka have even been pioneers in space biology (Ijiri 1995). We used medaka in our experiments because they are small, have a short generation time, and are iteroparous, spawning daily in the laboratory shortly after the photoperiod begins (Hirshfield 1980; Uematsu 1990; Grant et al. 1995b). We used wild-type and growth-enhanced transgenic medaka. Wild-type (hereafter W) medaka descended from wild fish captured in Japan (obtained from Pacific Aquatics, Chatsworth, CA, USA). Transgenic medaka (MtsGH-67, hereafter T; produced by Dr. William Muir, Purdue University), were engineered with a salmonid growth hormone gene construct (Devlin 1993; Chan and Devlin 1993; Devlin et al. 1994). We used RT-PCR to confirm expression of the transgene in earlier generations (Chapter 2) and used PCR to test for presence of the transgene.

Environmental treatments: Food availability and predation

We designed four environmental treatments to test the effects of food availability and predation on gene flow from transgenic to wild-type individuals (Table 1). Fish in Environment A were well-fed and free from the threat of predation: relatively sterile conditions like those found in many laboratories. Environment D, with low food availability and a threat of predation, was designed to be more similar to natural conditions.

We fed populations in high- and low-food availability treatments different amounts according to the mass and number of fish in each aquarium. We fed fish assigned to the

high-food treatment 10% of their total mass in flake food (O.S.I.) each day. We fed fish in the low-food treatment at a rate of 3% of their total mass per day. All tanks were fed flake food twice daily, with half of their ration delivered during each feeding, separated by at least one, but not more than eight, hour(s). Once a day, we fed 24-hour-old live brine shrimp larvae (*Artemia* spp.) to all tanks, at a rate of one drop per adult fish in the aquarium for high-food-availability treatments and one drop per two adult fish in the low-food-availability treatments.

We simulated predation with the head of a great blue heron (*Ardea herodias*) decoy (Sport Plast Decoy Company, Italy). In their native environments, medaka would likely encounter wading birds as a predator (Maeda 2001; Iguchi and Kitano 2008). Coincident with offering flake food to fish in predation-present treatments, we held the heron's head just above the water in the front half of the tank (Figure 1), striking the head below the water's surface 10-20 times during a five-minute simulation. These movements were similar to the predation simulation used by Jönsson et al. (1996), and like the hunting behavior of a heron (Kushlan 1976). To ensure that the fish reacted to the simulated predator instead of to the operator, we covered aquaria with an opaque fabric on the front. We exposed each population in the predation treatment to the simulated predator an average of once every ten days, or a total of 21 times per aquarium over the course of the experiment.[1]

[1] We also affixed a small dipnet to the end of the heron's beak and planned to assign any fish trapped in the net a randomly generated number between 1-4. Adult herons usually miss their prey only once every four attempts (Recher & Recher 1969). We therefore planned to release fish assigned a "1" unharmed back into the aquarium and remove fish assigned other numbers from the aquarium (to simulate death), after noting sex, weight, and genotype. However, no fish were caught in the net during predation simulations.

Experimental environment

We randomly assigned each of 24 113.5-L glass aquaria to one of the four environmental treatments. All aquaria were connected by a recirculating system, supplied by filtered well water flowing in at a replacement rate of approximately 20% system volume per day. All aquaria were located inside an insulated, photoperiod-controlled chamber, in which we maintained a photoperiod of 16:8 (l:d) and water temperature of 26°C to promote mating behavior (Shima and Mitani 2004). We separated each aquarium into a front and back section with a Styrofoam tank divider and, to encourage egg development and successful recruitment, provided a floating mesh basket to shelter fry from cannibalism and hanging spawning grass in the back of the aquarium (Figure 1).

Multi-generation population experiments

We used approximately 10-12 month old medaka of known genotype and sex to begin the population experiments. About six weeks before the beginning of the experiments, we tagged all "founder" fish with yellow fluorescent visible implant elastomer (VIE) tags (Northwest Marine Technology, Inc., Shaw Island, Washington, USA). These tags have high retention times and inflict low mortality even on small fish (Frederick 1997). We tagged transgenic fish on their left flank and wild-type fish on their right. Before tagging, we anesthetized fish with 80 mg/L MS-222 (Uematsu 1990) and, after tagging, placed fish in fresh water to recover, and we monitored all fish for infection at the tag site or tag loss after the tagging procedure.

In June 2008, we randomly chose four fish of each genotype and sex (n = 16) for stocking into each aquarium. We recorded weight and standard length (SL) for each individual. We censused each aquarium at regular intervals; we conducted the first five censuses on a biweekly basis, but reduced the frequency to monthly after observing signs of stress among the fish. During each census, we counted all fish in each aquarium, and fish with tags (founders) were identified by sex and genotype. We weighed the entire population of each aquarium to determine food levels. We thoroughly scraped algal growth from the sides of the aquaria, and siphoned all solid waste from all aquaria weekly. We ran the experiment for 210 days (30 weeks). At the end of the experiment, we again recorded weight and SL for each individual, and genotyped all fish remaining in the aquaria. We compared final transgene frequencies and population sizes among environments using Analysis of Variance (ANOVA), Tukey Honestly Significant Difference (HSD), and proportion (e.g., Newcombe 1998) tests.

Demographic simulation model

We created an age-structured population model to predict the effect of different environments on transgene frequency and population size. Because population experiments began with a female: male ratio of 1: 1, and because many fitness measures are relevant to only one sex, we modeled only females. We used a time step of one day and maximum individual longevity of 700 days (unpublished data, K. M. Pennington). The population model is based on fitness-component demographic models, similar to the net fitness model (e.g., Muir and Howard 1999), but was constructed as a matrix

population model (e.g., Starfield and Bleloch 1986; Caswell 2001) in Excel (Microsoft Office for Macintosh 2008).

The total female population size (assumed to be 0.5 of the total population) at time *t* is equal to:

$$N_t = \Sigma_d (\Sigma_j N_{j*d}), \quad \text{eqn 1}$$

where *d* is days of age, and *j* is female genotype, and sums are of females of all genotypes *j* at all ages *d* (Table 2).

The transgene frequency at time *t* is equal to:

$$N_{j=2} / N_t \quad \text{eqn 2}$$

Modeled populations at $t = 0$ began with 24 females of each genotype *j* evenly distributed between 301-306 *d* old; this mirrors the number, and approximate age, of females used in each environment in the population experiments. At each timestep ($t + 1$), the population of the previous age class was reduced by V_j, the daily viability for sexually mature adult females.

Beginning on $t = 1$, all sexually mature females ($N_{j, d>s}$) produced eggs according to:

$$N_{d=0} = \Sigma N_{j, d>s} * c_j /2 * r_k * m_k * o_{ijk}, \quad \text{eqn 3}$$

where $N_{d=0}$ is the number of eggs produced in one timestep destined to become females; c_j is the average daily fecundity for females of genotype j; r_k is the fertility rate of males of genotype k; m_k is the proportion of matings secured by males of genotype k; and o_{ijk} is the proportion of offspring of genotype i that result from a mating between a male of genotype k and a female of genotype j that will result in a female of genotype j. Seven days after eggs are produced, they hatch according to h_{jk}, the proportion of fertile eggs produced by a female of genotype j and a male of genotype k that hatch:

$$N_{d=1} = N_{d=0} * h_{jk}. \qquad \text{eqn 4}$$

Between $d = 2$ and $d = s_j$, the juvenile population is reduced at each timestep according to v_j, the daily viability for juvenile fish of genotype j. At $d = s_j + 1$, the entire age class is recruited into the adult female age class, and those females begin to produce eggs and are subject to the V_j viability rate. Overall proportions of fish surviving (pv) were transformed to daily viabilities with the equation

$$v_j (\text{or } V_j) = e^{((\log (pv)/ (d(t+1)) - d(t)))} \qquad \text{eqn 5}$$

(e.g., Muir 2002).

Most of the values for the model's parameters were derived from fitness component data from previous experiments in which these traits were measured under the same four

environmental conditions in the same recirculating water system (Chapter 3), except: (1) data for proportion of fish of genotype *i* resulting from mating type *jk* were derived from the known genotype and parentage of fish used in age at sexual maturity measurements in the four different environments (unpublished data, K. M. Pennington), and (2) daily adult viability values, which were derived from the mortality rate observed during this experiment (Table 3).

Because our fecundity estimates were based on fish 300-360 days old, we corrected the fecundity of subadult fish, as in other fish population models (e.g., Hutchings 2005). Because fecundity and size are closely related in fish in general (e.g., Bagenal 1978; Blueweiss et al. 1978; Jobling 1995), and correlated in medaka (Chapter 3), we used the general logistic relationship (Schnute and Richards 1990) to estimate fecundity from sexual maturity to age 300 days,

$$y = 1/ (1 + \alpha e^{-ad}), \qquad \text{eqn 6}$$

where y is the proportion of adult fecundity estimated for a fish of age d. We adjusted parameters $a = 0.8$ and $\alpha = 5.1$ to obtain an approximate fit to our data. Because y is in the form of a proportion of a maximum value, we used the equation to predict values for y at nine different time points ($d = s_j$ to $d = 90$, and in 30-day intervals thereafter to $d = 300$;) and used these values, multiplied by the value for c_j in each environment to calculate values for total fecundity at eight subadult age stages.

We ran deterministic and stochastic versions of the population model using

methods for incorporating parameter variability − a common form of uncertainty − into ecological risk assessment models (Burgman 2005; Hayes et al. 2007). In the stochastic model, we addressed uncertainty about fitness trait parameters using a Monte Carlo simulation (e.g., Regan et al. 2003) varying adult and subadult fecundity, male fertility, mating probability, juvenile viability and hatch proportion over a beta distribution (Table 3). We used the beta distribution because it allowed us to vary fitness trait values over a continuous distribution with upper and lower bounds (Bolker 2008). This distribution is commonly used to model life history components in animal demographics (Emlen and Pikitch 1989; Benton and Grant 2000; McGowan and Ryan 2009). We calculated the maximum value for fecundity as 1.1*(maximum observed value), and 0 as the minimum. We set minima and maxima for all other fitness traits at 0 and 1, respectively. We estimated alpha and beta shape parameters for each beta distribution using method of moments estimation (NIST/SEMATECH 2006). Test runs of the model revealed several fitness estimates that caused the model to fail; we used manual iteration to slightly alter the mean and/or SD of these values to coerce the Monte Carlo simulations to return real numbers (Table 3). We ran both the deterministic and Monte Carlo model using parameters from each environment for $t = 1000$ days. We ran 1000 iterations of the Monte Carlo simulation, and for each iteration recorded the population size and transgene frequency at $t = 210$ and $t = 1000$.

We used the R statistical package to perform analyses and to produce graphs (R Development Core Team 2008; Sarkar 2008; Warnes 2009). We used Excel (v. 12.2.3, Microsoft Office for Mac 2008) to run the demographic model, and Risk Engine to

perform Monte Carlo simulations (Turner 2009).

Results

Multi-generation population experiments

Populations in Environments A and B increased at a rapid rate at the beginning of the experimental period, but as new recruits suffered mortality, the population size decreased nearly to the level of populations in Environments C and D (Figure 2). Although the total biomass of fish increased in Environments A and B relative to C and D, especially during days 50-200 of the experiment (Figure 3a), the per capita weight of fish was not very different between environments (Figure 3b). Final adult sex ratios were not different between environments (Tukey HSD, all adj. $P > 0.226$), and the final average proportion of adult females in each environment was within one standard deviation of the initial rate of females, 0.5 (mean ± SD; A: 0.587 ± 0.177; B: 0.492 ± 0.232; C: 0.450 ± 0.069; D: 0.664 ± 0.221).

Founder T fish suffered a higher rate of mortality than founder W fish during the experiment (Figure 4). All founder T fish had died off by the 168-day census in Environment D and by 196 days in Environments B and C. At the end of the experiment, two populations (out of six) in Environment A still had founder T fish, and all populations in Environment A had from 2-11 total T fish (including founders and subsequent generations). Though no founder T fish persisted in the other environments at the experiment's end, at least one T fish remained in five of six populations in Environment B, T fish still persisted in two populations in Environment C, and a single T

fish survived in one population (out of six) in Environment D. At the end of the experiment, total population size decreased from Environments A to D, as did the total number of T fish (Figure 5). There were no significant differences among Environments with regard to total final population size (Tukey HSD, all adj. $P > 0.192$), adult final population size (Tukey HSD, all adj. $P > 0.147$), or total W final population (Tukey HSD, all adj. $P > 0.518$). However, the total T final population was significantly greater in Environment A than in all other Environments (Tukey HSD; A-B adj. $P = 0.023$, A-C adj. $P = 0.006$, A-D adj. $P = 0.002$).

Final transgene frequencies were all significantly less than 0.5, the starting transgene frequency (Figure 6) (proportion tests: A: $\chi^2_{(df=1)} = 4.381$, $P = 0.036$; B: $\chi^2_{(df=1)} = 26.179$, $P < 0.001$; C: $\chi^2_{(df=1)} = 20.471$, $P < 0.001$; D: $\chi^2_{(df=1)} = 25.255$, $P < 0.001$). Environmental treatment was a significant predictor of final transgene frequency (ANOVA, $F_{(3, 20)} = 9.32$, $P < 0.001$). The final transgene frequency in Environment A was significantly greater than that in Environments C or D (Tukey HSD, both adj. $P < 0.005$).

Demographic simulation model

The deterministic model predicted that population size would grow exponentially in all environments, with the final population size highest in Environment A and lowest in Environment C (Figure 7a). The predicted transgene frequency at $t = 210$ was highest in Environment A: 0.897; predicted transgene frequencies were 0.335, 0.425, 0.339 in Environments B, C, and D, respectively (Table 4). However, the modeled transgene

frequency was still fluctuating at $t = 210$, particularly in Environments B, C, and D (Figure 7b). At $t = 1000$, the deterministic model predicted transgene frequencies of 0.925, 0.264, 0.535, and 0.394 in Environments A, B, C, and D respectively (Table 4).

As with the deterministic model, in Monte Carlo simulations, all modeled populations grew exponentially. Predicted population sizes at $t = 210$ in all Environments were greater than the population sizes we observed in our population experiments (Figure 8). At $t = 1000$ days, predicted population size in Environment A ranged much higher than in any other environment (Figure 9). The untransformed predicted population size in Environment A was significantly greater than in any other environment (Tukey HSD, all adj. $P < 0.001$), and Environment B had a significantly greater population prediction than Environments C or D (Tukey HSD, both adj. $P < 0.001$); predicted population size was not different between Environments C and D (Tukey HSD, adj. $P = 1.000$).

Predicted transgene frequencies in Environment A were higher than in all other environments at $t = 210$ and $t = 1000$ (Figure 10). Mean predicted transgene frequency at $t = 1000$ was 0.933 in Environment A, 0.290 in Environment B, 0.401 in Environment C, and 0.478 in Environment D (Table 4). All Monte Carlo predicted transgene frequencies, at both t, were significantly different from one another (Tukey HSD, all adj. $P < 0.020$).

Discussion

We expected that the final population and size and transgene frequency would decrease from Environments A to D, as environmental conditions became poorer. Indeed, the observed final transgene frequency did follow this trend. However, though

population sizes were higher in Environments A and B during the middle of the experiment, all environments had similar population sizes at the experiment's end. This was likely due to density-dependent effects, as the volume of experimental aquaria would prevent unchecked exponential population growth, as occurred in our modeled populations.

The final observed transgene frequency in Environment A was greater than in Environments C and D, and the total number of transgenic fish was greater in Environment A than in any other environment. Because Environment A had the most sterile culture conditions, it least resembles an environment that transgenic fish might encounter in nature. This illustrates the importance of incorporating variation in environmental conditions, like food availability and predation, which can have effects on fitness component values (Chapter 3), when using confined experiments or quantitative models to predict the fate of transgenes in wild populations.

Both the deterministic and Monte Carlo models yielded predictions of transgene frequency over multiple generations that were partly consistent with our observed results, in that the final transgene frequency in Environment A was higher than all other environments. Monte Carlo model predictions of transgene frequency most closely matched our results in Environment B, whereas in Environments C and D the predictions began to drift away from our observed values (Figure 6).

All model predictions failed to correctly predict the relative ranking of environments with regard to observed transgene frequency. We observed a decrease in the proportion of transgenic fish from Environment A to Environment D whereas Monte

Carlo simulations (at $t = 210$ and $t = 1000$) predicted that the transgene frequency in Environment D would be greater than that of Environment B or C (Figure 6; Table 4), and the deterministic model predicted the second-highest transgene frequency would be in Environment C (Figure 7b; Table 4). All predictions overestimated the transgene frequency in Environments A and D at $t = 210$, and, interestingly, the Monte Carlo simulations had very low variation around the mean prediction in Environment A. Conversely, the transgene frequency that we observed in Environment D had the lowest observed standard deviation of all environments, but Monte Carlo model predictions had the largest spread in Environment D (Figure 10). Had we only measured fitness traits in Environment A, and modeled gene flow based on those measurements, we (and risk assessors) might have been misled by this apparently precise model prediction of high transgene frequency.

In the Monte Carlo model, we varied a number of fitness traits around observed values to explore the range of model predictions resulting from empirically measured uncertainty in fitness trait parameters. Previous deterministic gene flow models (Muir and Howard 1999; Hedrick 2001; Muir and Howard 2001) have varied parameter values by an arbitrary amount to explore the relationship between parameters and predictions. We varied the parameters male fertility, male mating success, female fecundity, and juvenile viability, all of which are moderately influential parameters in deterministic net fitness models (Muir and Howard 2001). We also measured, and included in our model, different hatch probabilities for offspring of each parental cross in each environment, a fitness-related trait that was not included in previous versions of the net fitness model. We did

not vary two fitness values in our Monte Carlo model: adult viability and age at sexual maturity. Because our estimates of daily adult viability values were relatively high (0.936-0.997), and because fitness component models are relatively insensitive to this parameter (Muir and Howard 2001) we chose not to vary this parameter. Age at sexual maturity, on the other hand, may have a relatively large impact on predictions of transgene frequency (Muir and Howard 2001); because our model was constrained by the matrix structure in which juvenile females were recruited to a sexually mature age class on a particular day, this parameter could not be varied using Monte Carlo simulation. However, this constraint is mitigated by the fact that we reduced the fecundity of subadult females according to eqn 6; in our model, the reproductive impact of newly-sexually mature females was not as great as in previous models (i.e., Muir and Howard 2001).

Though models can be a useful way for population biologists to understand demographics, they should always be accompanied by the caveat that they are not an exact reflection of reality (Levins 1966). However, it is difficult for many biologists and risk assessors to accept the abstraction that is necessary to model nature, particularly when models attempt to predict the outcome of a complex process such as gene flow. Some have argued that, because of the complexity of natural environments and processes therein, even the combined analysis of experiments and models, as we have done, is insufficient to make reliable predictions about the invasiveness of a transgenic organism in nature (Kareiva et al. 1996). Short of abandoning all use of risk assessment, a tenable response to this critique is to explicitly identify and treat the sources of uncertainty in all

parts of a risk assessment (Hayes et al. 2007). Our results are a major step towards this end in that we measured fitness traits on transgenic organisms in a number of different environments and simulated uncertainty about life-history parameters on the outcomes of multigenerational, population-level processes.

The tendency to tune models to match experimental data, achieving "good results for the wrong reasons," is another reason to be wary of model predictions (Scheffer and Beets 1994: p. 116). Our model parameters, therefore, were based on empirical measurements, and were not altered to match predicted results to our observed results. Still, debate lingers on how best to characterize models in relation to experimental results. The term "model validation" has often been used to imply agreement of model predictions and experimental observations. Some authors have argued that the concept of validation runs counter to the scientific method (Reckhow and Chapra 1983) while others contend that validation may be an appropriate term under certain circumstances (Rykiel 1996). However, we prefer to evaluate our results in terms of "model confirmation," i.e., observations that "confirm" a model's predictions do not mean that a model is true, only that it is not wrong (Oreskes et al. 1994). To ascertain if our model is not wrong with respect to our observed data, we used boxplots (Figure 10), as they are informative nonparametric methods for comparing multiple datasets (Reckhow and Chapra 1983). These suggest that our model is confirmed by observations in Environment B, as the interquartile range of our observed and predicted (at $t = 1000$) transgene frequencies do overlap (Reckhow and Chapra 1983). Environment C data nearly confirmed model predictions, as the whiskers of the predicted transgene frequency values at both $t = 210$

and $t = 1000$ overlap with the interquartile range of observed values (Figure 10). Data for Environments A and D, in contrast, did not confirm model predictions, though one outlier observation did fall within the range of the whiskers for predictions in Environment D. This may suggest that models based on fitness data taken in relatively sterile environments (like Environment A) or relatively harsh environments (like Environment D) will be less able to predict gene flow in populations exposed to those extreme environments.

Model limitations and future research

All models have limitations, because a single model cannot have precision, realism, and generality all at once (Levins 1966). Our model was designed to test whether measuring fitness traits under a given environment can help predict the outcomes of population processes in the same environment, and we prioritized realism by using parameters experimentally estimated in different environments and by introducing stochasticity. We made several simplifying assumptions regarding both the experimental and modeled populations: there was only one invasion event, populations were exposed to the same environmental conditions throughout the experiment, and changing sex ratio and population density did not affect fitness trait values.

Our model included only one invasion event, at a relatively high rate (0.5 transgene frequency). In natural systems, wild populations of fish may be subject to chronic invasion by farm fish as well as larger influxes resulting from failures in containment (Naylor et al. 2005). High levels of immigration of invasive species have been found to

hasten displacement of and introgression into native populations (Huxel 1999). Future models and confirmation experiments could simulate this by allowing additional transgenic fish to "escape" into the experimental or modeled population at random intervals.

Though we used several different environmental conditions, spatial heterogeneity in the environment (Tyler and Rose 1994) would also affect gene flow processes in highly mobile species like salmon, which might encounter environments like our Environment A and like our Environment D during different stages of their life cycle. Models and experiments could address spatial heterogeneity by measuring fitness traits under relatively rich or poor environmental conditions, relevant to the life history of the species in question. Temporal variation in environments could also be simulated in models and confirmation experiments.

We assumed that modeling only females was sufficient and that the sex ratio was relatively stable. It may be necessary to model both sexes in fish species where sex ratio influences fitness and life history traits. In medaka, male competition for mates increases as male: female sex ratios increase (e.g., Grant et al. 1995a). We tested male mating advantage under a static sex ratio of two males: one female (Chapter 3) and our model implicitly assumed that these mating probabilities would persist regardless of the operational sex ratio in the population (the same assumption is implicit in Howard et al. 2004). Future research could measure fitness trait values such as relative mating advantage over a range of sex ratios, and incorporate any effects into a demographic model.

Density dependence has also been found to influence gene flow and introgression in models (e.g., Aikio et al. 2008b; Bax and Thresher 2009). Our model did not include density dependence, though this was evident in our population experiments because populations were constrained by the carrying capacity of the aquaria and could not grow exponentially as did our modeled populations. Though our fitness trait data were collected under lower densities than those in the gene flow experiment (except juvenile viability), our model assumed no effect of density on those trait values. This could have resulted in overestimates of fecundity and fertility that were reflected in our models' predictions of exponential population growth. Similar to addressing the effect of sex ratio on mating advantage, investigators could measure fitness traits under a variety of densities, and use those data to parameterize modeled populations of different densities. Confined population experiments are likely to impose different density dependent constraints on population growth than the natural ecosystems into which transgenic fish would escape. Therefore, research to better understand if, and how, density dependence alters fitness trait values could improve the applicability of risk assessment models to natural populations.

Conclusion

The differences between confined experimental results and outputs from the Monte Carlo model simulations underscore the importance of incorporating natural variation in fitness related traits into predictions of gene flow to inform ecological risk assessments of transgenic fish. We found that fecundity, fertility, juvenile viability, and mating

advantage, when varied randomly over ranges actually observed in our experiments, led to predictions of transgene frequency that did overlap with observed results in some environments, and predictions that were not confirmed by our experimental observations in the most extreme environments. Our results also demonstrate the potential danger of basing gene flow risk assessments on measurements of fitness traits from only a single environment – particularly extreme environments like A or D. Furthermore, our findings advise against using fitness trait data to parameterize a deterministic model, instead of a stochastic model, and making risk assessment decisions based on those model outcomes. We strongly recommend building uncertainty analysis into gene flow models at least by incorporating parameter variability (Regan et al. 2003; Hayes et al. 2007), and confirming model predictions with data collected under relevant environmental conditions, before using such models to inform ecological risk assessments.

Table 1. Four different environmental conditions generated by crossing two levels of the factors food availability and simulated predation.

		Simulated predation	
		absent	*present*
Food availability	*high*	A: "laboratory" environment	B: predation
	low	C: limited food	D: "natural" environment

Table 2. Model parameters.

Symbol	Description
c_j	Mean daily egg production by females of genotype *j*
r_k	Proportion of eggs fertilized by males of genotype *k* (measured using W females, assumed same for both genotypes)
s_j	Age of sexual maturity for females of genotype *j*
m_k	Proportion of matings obtained by males of genotype *k* (measured using W females, assumed same for both genotypes)
o_{ijk}	Proportion of offspring of genotype *i* that result from a mating between a male of genotype *k* and a female of genotype *j* that will result in a female of genotype *j*
h_{jk}	Proportion of eggs resulting from a mating between female of genotype *j* and male of genotype *k* that successfully hatch
v_j	Daily viability for juvenile ($d < s_j$) females of genotype *j*
V_j	Daily viability for adult ($d > s_j$) females of genotype *j*
d	Age (in days)
t	Time (in days)
i	Genotype of offspring (1 = W, 2 = T)
j	Genotype of female (1 = W, 2 = T)
k	Genotype of male (1 = W, 2 = T)
y	Proportion of mean fecundity attained by subadult females, $s_j < d < 300$

Table 3. Means ± SD for fitness measurements of different genotypes measured in environments A through D (Chapter 3), used to parameterize the demographic model (maxima used to derive parameters for beta distributions in parentheses).

Fecundity (c_j) (these ten day totals were divided by 20 to obtain daily production of eggs destined to be female fish for use in model; observed maxima in parentheses):		
Female genotype:	W	T
A	168.86 ± 140.94 (365)	293.75 ± 121.52 (413)
B	245.88 ± 105.15 (343)	322.43 ± 143.34 (593)
C	131.29 ± 83.67 (234)	138.50 ± 79.80 (203)
D	165.00 ± 114.29 (390)	201.60 ± 19.78 (217)

Daily fecundity of newly-sexually mature females (divided by 2 to obtain number of eggs destined to be female fish for use in model; observed maxima in parentheses):		
Female genotype:	W	T
A	5.00 ± 3.597 (14)	5.00 ± 2.370 (12)
B	9.30 ± 6.000 (20)	7.68 ± 4.233 (19)
C	7.29 ± 6.462 (23)	4.15 ± 2.612 (11)
D	4.50 ± 2.771 (11)	4.46 ± 2.519 (11)

Fertility (r_k):		
Male genotype:	W	T
A	0.943 ± 0.142	0.951 ± 0.074
B	0.947 ± 0.110	0.988 ± 0.024
C	0.988 ± 0.020	0.996 ± 0.005[a]
D	0.996 ± 0.007	0.921 ± 0.142

a. To derive beta distribution parameters, used sample SD × 2 = 0.010

Table 3, continued:

Mating probability (m_k):		
Male genotype	W only	T only
A	0.3125 ± 0.239	0.6875 ± 0.239
B	0.8542 ± 0.172	0.1458 ± 0.172
C	0.8125 ± 0.239	0.1875 ± 0.239
D	0.5625 ± 0.427	0.4375 ± 0.427

Proportion (offspring of genotype *i* | mating type *jk*) (o_{ijk}):				
Parental genotypes	WW	WT	TW	TT
W offspring	1.0	0.14	0.13	0
T offspring	0	0.860	0.87	1.0

Proportion of fish hatching from fertile eggs (h_{jk}); standard deviation (SD) is based on all parental genotypes in an environment:					
Parental genotype	WW	WT	TW	TT	SD
A	0.974	0.963	0.892	0.916	0.042
B	0.860	0.952	0.973	0.813	0.102
C	0.692	0.959	0.847	0.848	0.108
D	0.834	0.922	0.912	0.955	0.057

Table 3, continued:

Proportion of fish surviving from hatch to sexual maturity; standard deviation (SD) is based on all parental genotypes in an environment (used eqn 5 to derive daily juvenile viability, v_j):

Parental genotypes	WW	WT	TW	TT	SD
A	0.833	0.950	0.967	0.867	0.082
B	0.567	1.000[b]	0.900	0.817	0.176
C	0.444	0.933	0.983[c]	0.767	0.214
D	0.633	0.900	0.733	0.905	0.143

Proportion of founder fish surviving during experiments (used eqn 5 to derive daily adult viability, V_j):

Adult genotype	W	T
A	0.417	0.042
B	0.396	0.000
C	0.458	0.000
D	0.521	0.000

Age at maturity (s_j):

Female genotype	W	T
A	55.556 ± 3.598	53.407 ± 2.370
B	59.900 ± 6.001	58.780 ± 4.233
C	72.143 ± 6.462	70.212 ± 2.612
D	67.778 ± 2.771	68.250 ± 2.519

b. To derive beta distribution parameters, used mean reduced to 0.995, and sample SD × 0.5 = .090

c. To derive beta distribution parameters, used sample SD × 0.5 = 0.107

Table 4. Observed (Obs) and predicted (D = deterministic, MC = Monte Carlo) transgene frequencies (± SD) in Environments A-D, at different times *t*.

Env.	Obs. (*t* = 210)	D (*t* = 210, 1000)	MC (*t* = 210)	MC (*t* = 1000)
A	0.332 ± 0.100	0.897, 0.925	0.912 ± 0.024	0.933 ± 0.018
B	0.169 ± 0.134	0.335, 0.264	0.364 ± 0.052	0.290 ± 0.068
C	0.086 ± 0.134	0.425, 0.535	0.312 ± 0.061	0.401 ± 0.091
D	0.017 ± 0.041	0.339, 0.394	0.376 ± 0.156	0.478 ± 0.207

Figure 1. Configuration of each tank used in population experiments.

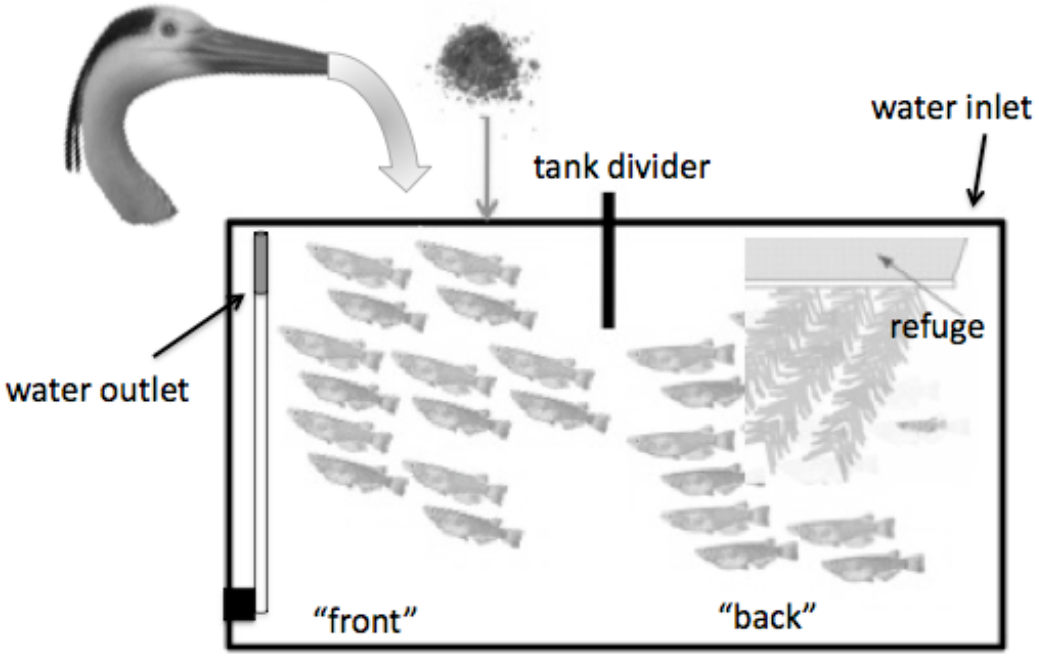


Figure 2. Total population in Environments A-D during the course of the experiments.

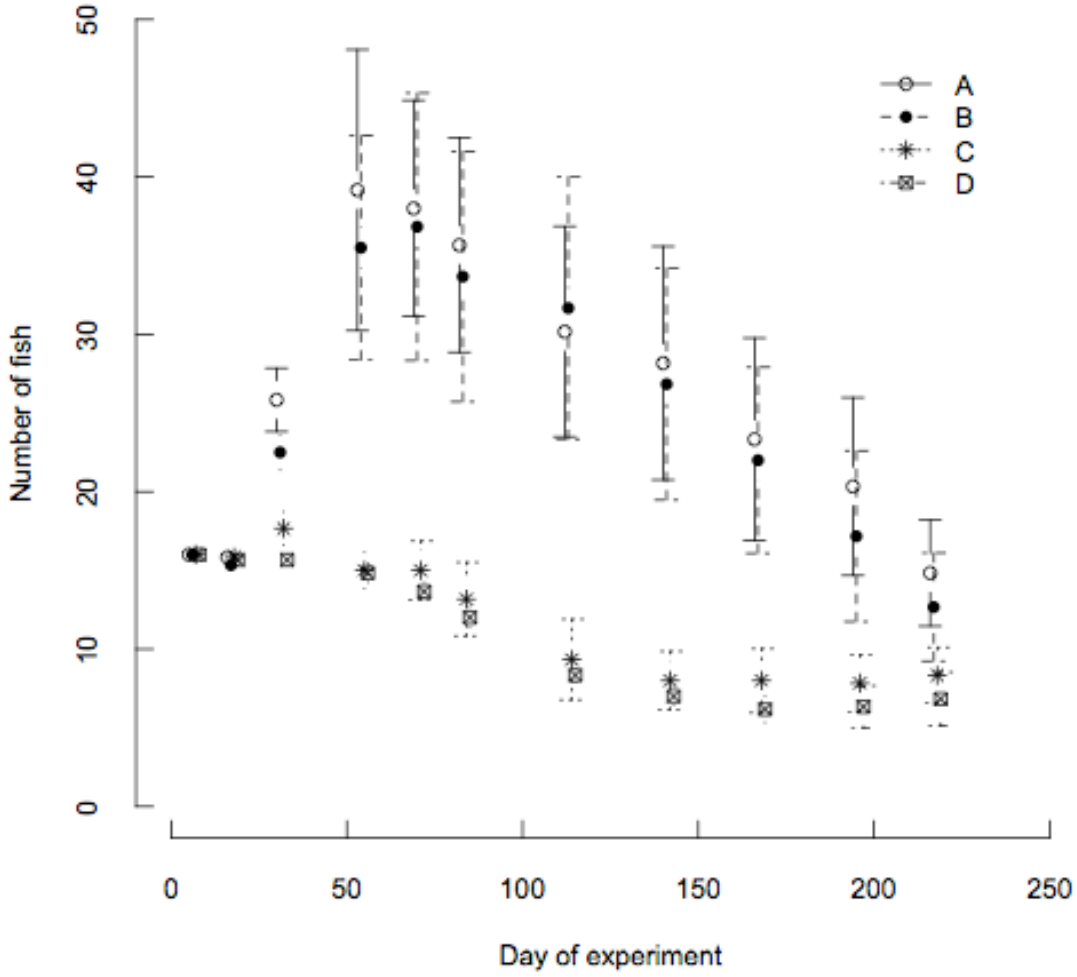
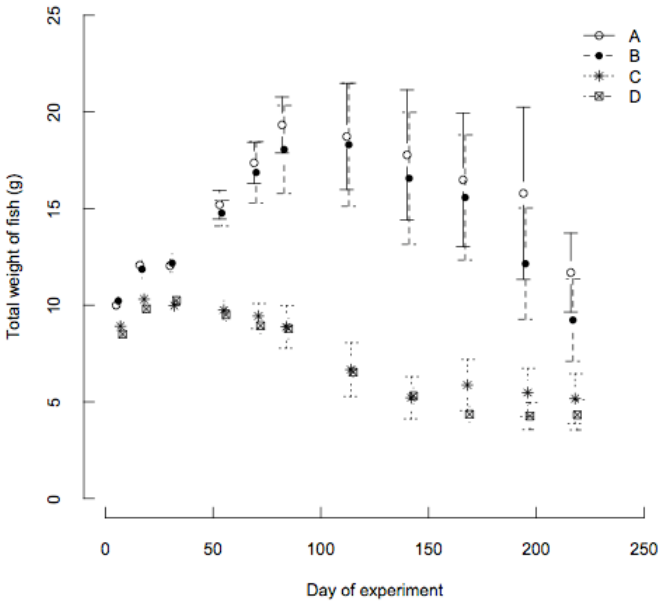


Figure 3. (a) Total biomass of fish in four different environments during the experiment was greater in Environments A and B, but (b) per capita weight of fish did not differ among environments.

a



b

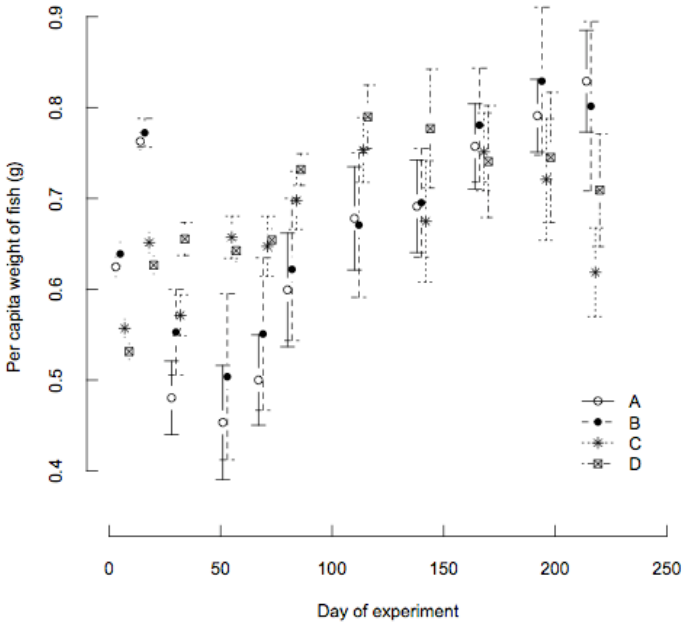


Figure 4. Transgenic founder fish died at a higher rate than wild-type fish, across all environments.

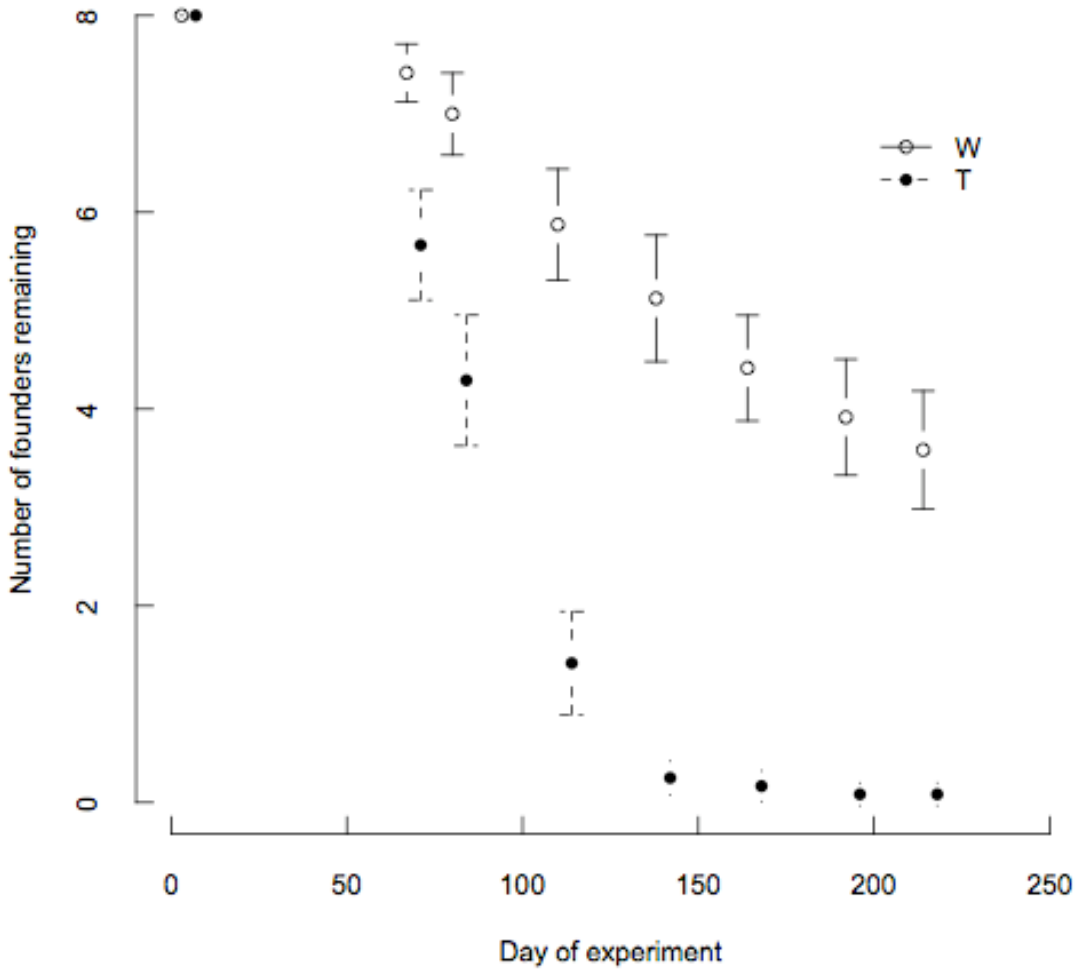


Figure 5. Total final population size in experimental tanks decreased from Environment A to D; and the number of total T fish remaining at the end of the experiment followed the same pattern. Dotted line denotes initial numbers of fish at $t = 0$. * Denotes number of T fish at $t = 210$ was significantly greater in Environment A than in B, C, or D (Tukey HSD, all adj. $P < 0.023$).

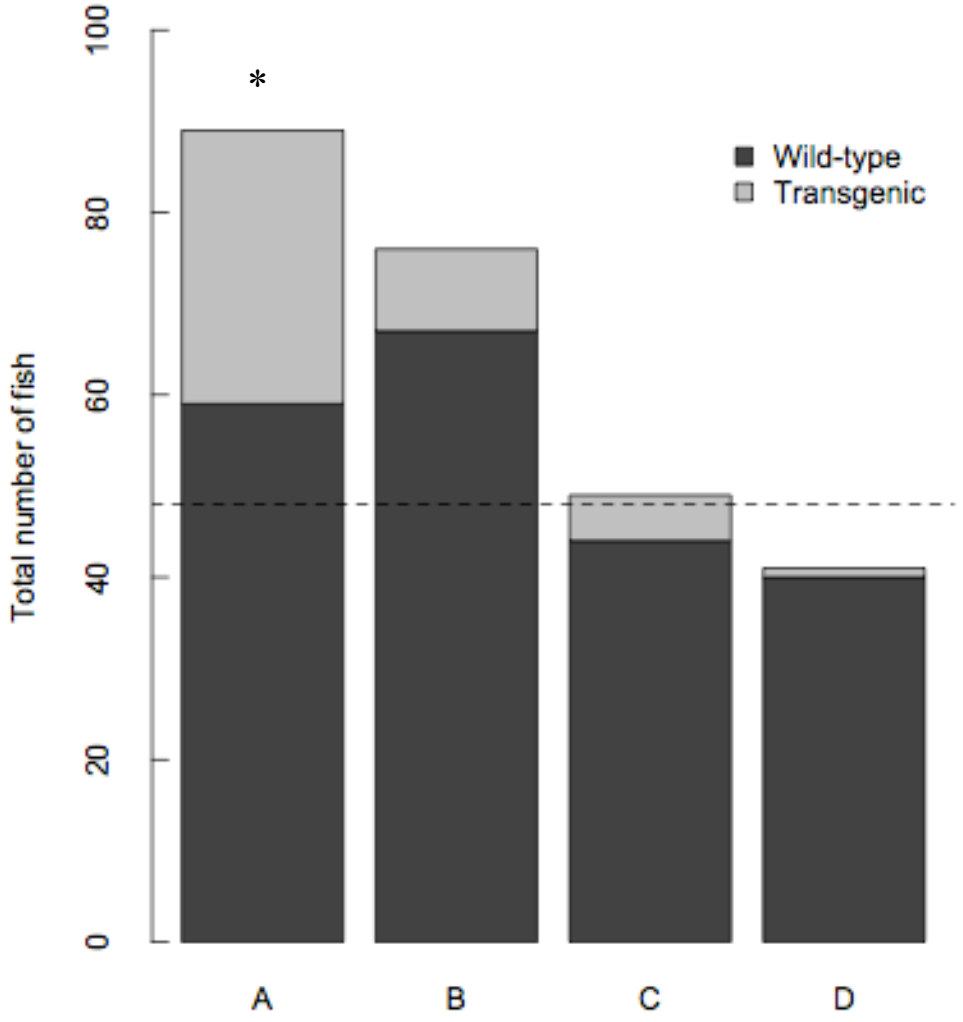


Figure 6. Observed and predicted (with Monte Carlo model) final transgene frequencies in Environments A-D. Observed transgene frequency at $t = 210$ was significantly greater in Environment A than in Environments C and D (Tukey HSD, both adj. $P < 0.005$). Error bars = 1 SD. Dashed line is the starting transgene frequency, 0.5.

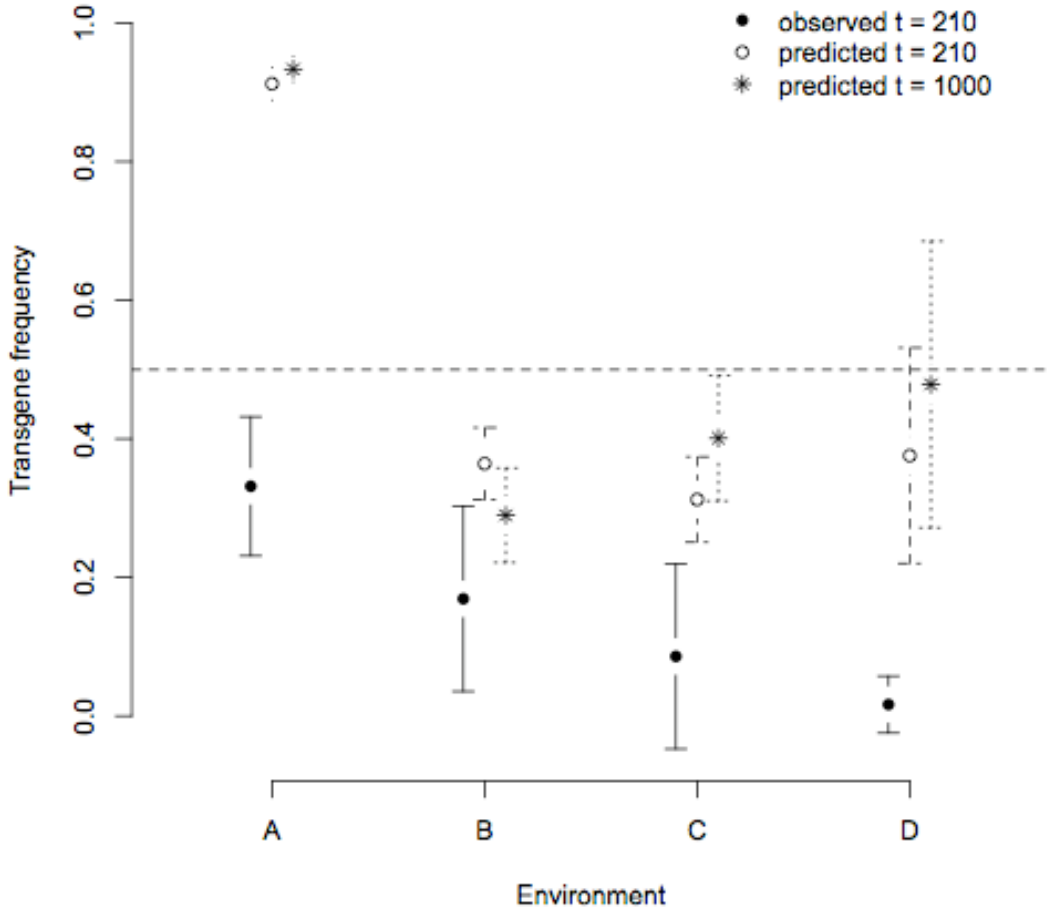
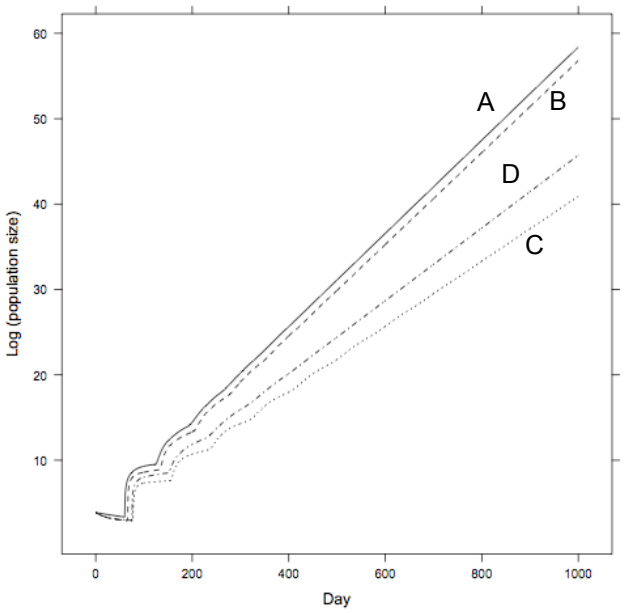


Figure 7. Deterministic model predictions of (a) log population size and (b) transgene frequency in Environments A-D.

a.



b.

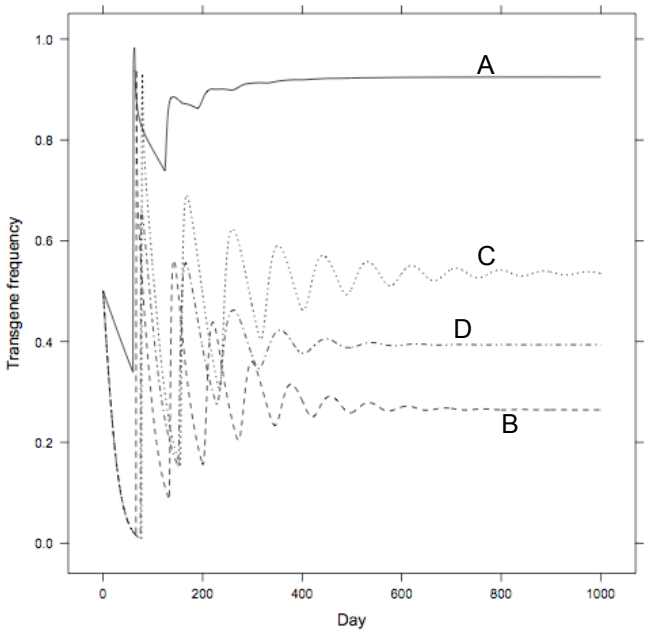


Figure 8. The stochastic model's predicted log population sizes at $t = 210$ were much higher than observed values. Dotted line denotes initial log population size at $t = 0$. Error bars = 1 SD (SDs for predicted log population size are too small to display).

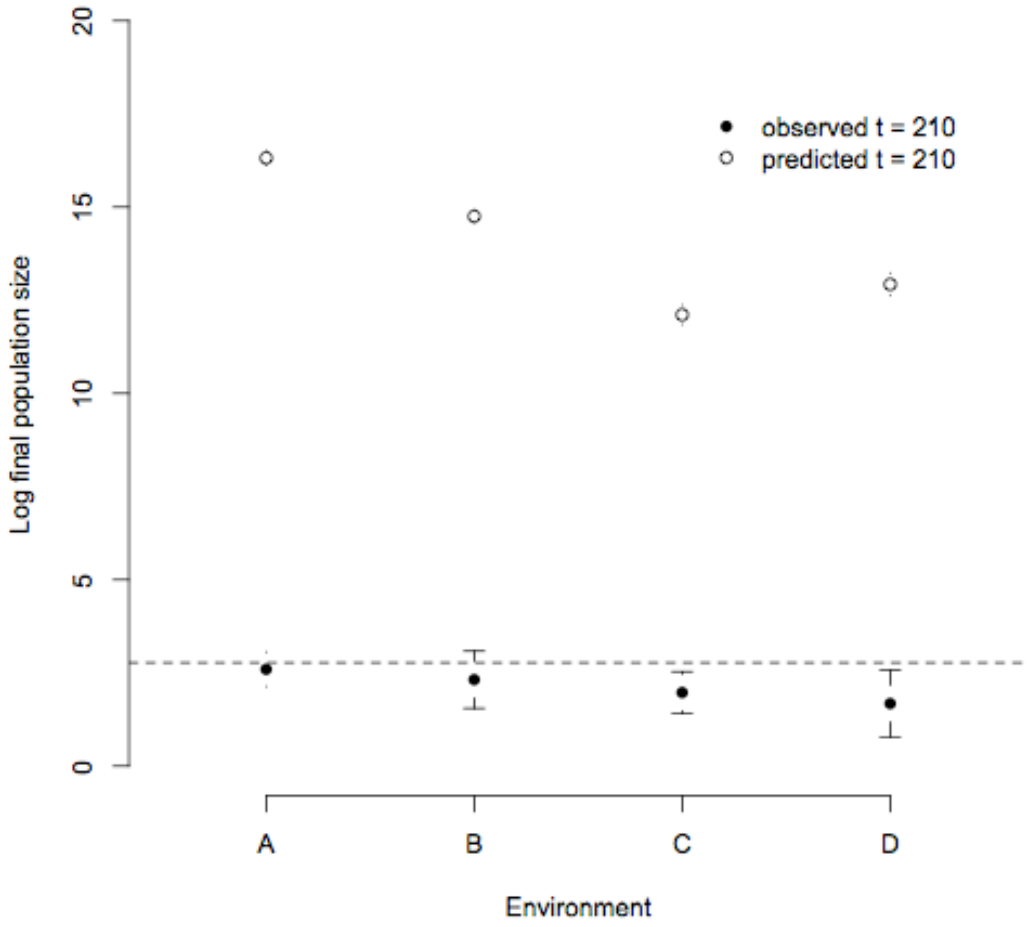


Figure 9. Frequency distributions of population sizes predicted by Monte Carlo simulations at t = 1000 in Environments A-D (note different scales on x-axes).

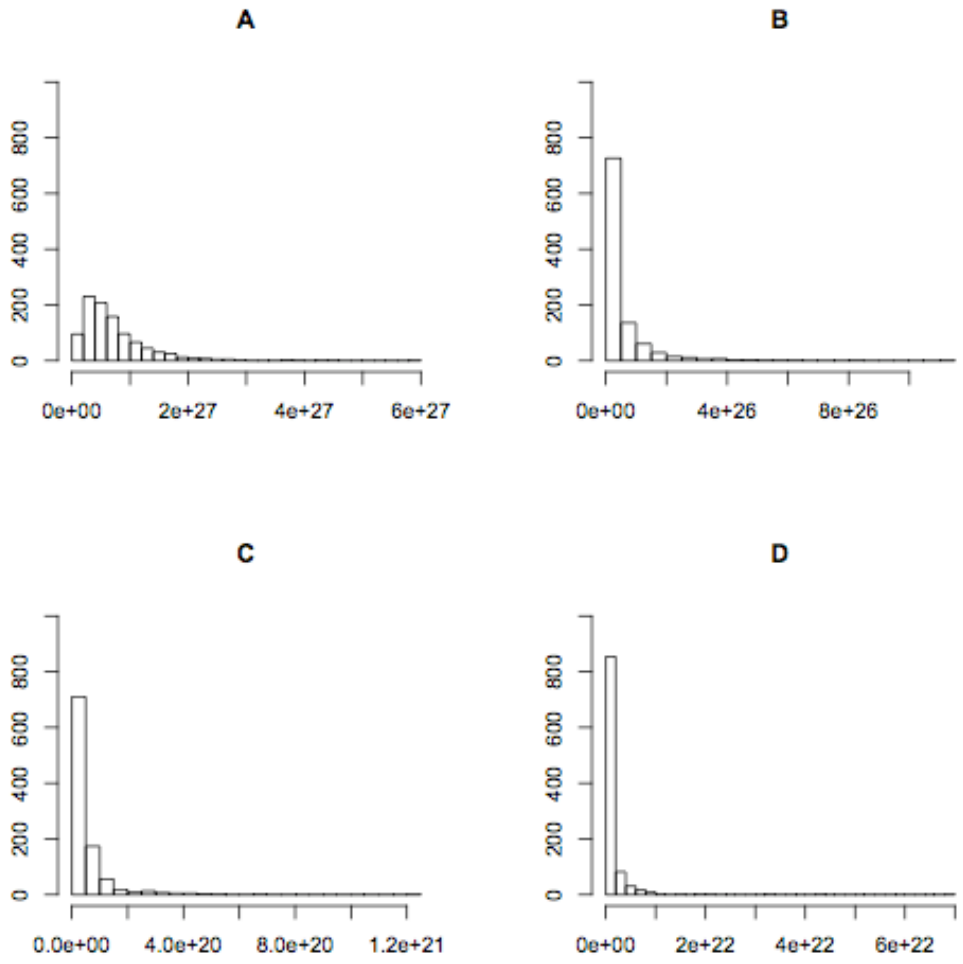
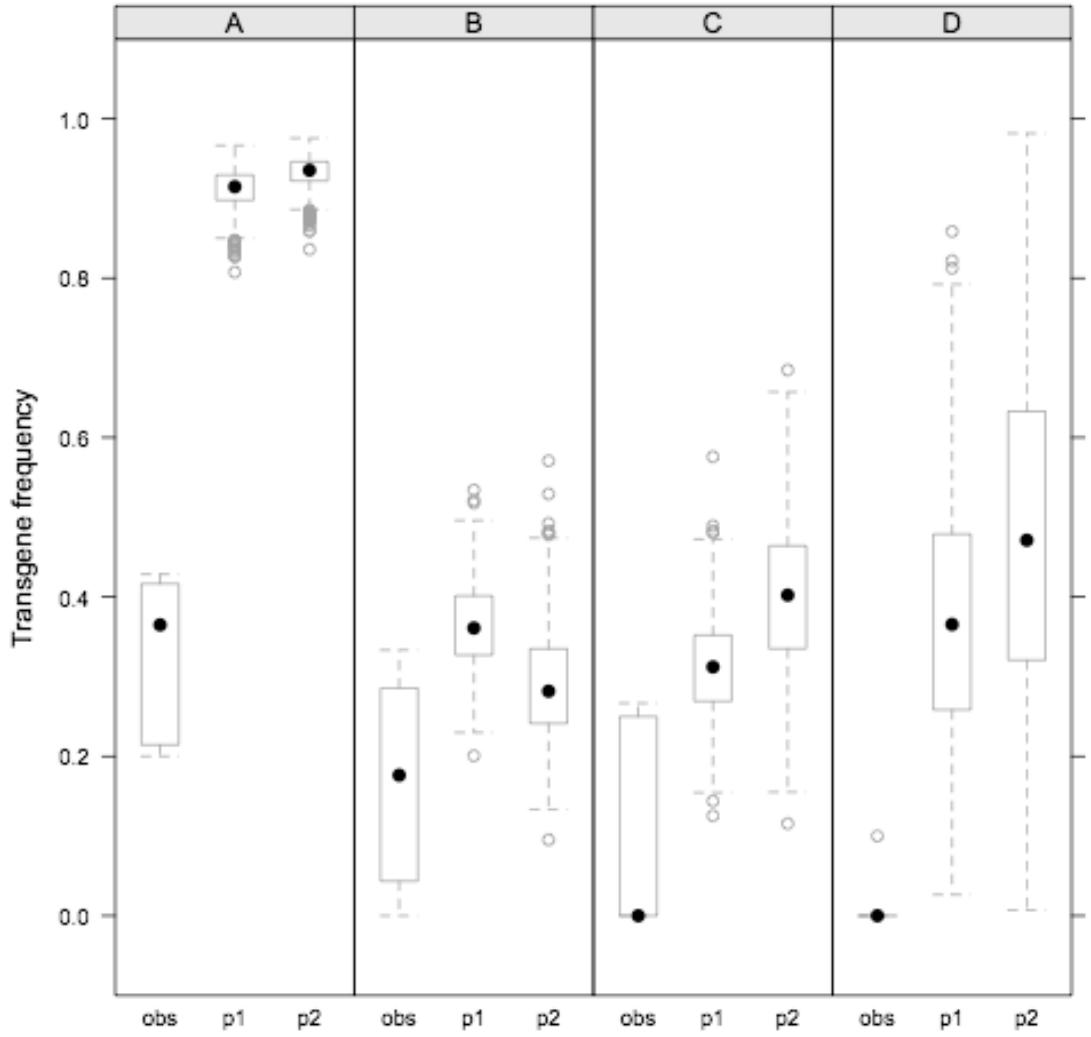


Figure 10. Boxplots of transgene frequencies in each environment: observed at $t = 210$ (obs), predicted by Monte Carlo model at $t = 210$ (p1), and predicted by Monte Carlo model at $t = 1000$ (p2). Solid dots are median values; box encompasses upper and lower quartile. Open dots are outliers, beyond the whiskers (which encompass values within 1.5 × the interquartile range).



CHAPTER 5

Concluding remarks

In Chapter 2, I gained confidence in the potential of fitness component data to predict transgene frequency in a population, because observed fitness trait data was not inconsistent with the results that we observed in mesocosm populations. In Chapters 3 and 4, I chose to incorporate different levels of food availability and predation into fitness and gene flow experiments. I found that environmental conditions did affect fitness trait values in both genotypes, and that some fitness traits were more influenced by environment than genotype. When I tested gene flow from transgenic fish under different environments, I found that transgenic fish were most successful in the environment with ample food and no predation. Modeled populations, parameterized with fitness trait values measured in the same environments, also indicated that high transgene frequencies were most likely in the high food availability, no predation, environment. However, not all model predictions were confirmed by our experimental observations.

Taken together, my results indicate that using fitness component data to predict the risk of gene flow from transgenic fish may be a promising approach. However, future models and confirmation experiments can help risk assessment scientists to better understand the relationships between relative fitness and gene flow, and to test those relationships in relevant environments, before applying predictions from models alone to decisions about the risk of gene flow from transgenic fish. Also, future modeling experiments should incorporate uncertainty about fitness parameters and environmental conditions, in order to reflect the variability inherent in natural systems. Though models can never be "correct," they can help to inform risk decision-making regarding transgenic

fish, and models that reflect uncertainty in parameters can provide better information about the range of impacts to natural populations and ecosystems.

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